HISTOCHEMICAL STUDIES OF NASOPHARYNGEAL CARCINOMA AND ASSOCIATED INTRAEPITHELIAL CHANGES

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CONTENTS

SUMMARY

Despite the World Health Organisation's recommendations made in 1978, there is still a lack of concurrence on the histopathological classification of the carcinomas of the nasopharynx (NPC). A major reason for this is that the exact histogenesis of the tumours is not understood. This study, therefore, was commenced in order to try and define the histochemical properties of the epithelia in the normal and in the abnormal nasopharynx, including those showing both intraepithelial and malignant changes.

The aim was to do as much of the work as possible on paraffin processed nasopharyngeal tissues, in order that a retrospective study could be undertaken, and so that the results, if significant, could be applied to routine diagnostic work.

Conventional histochemical techniques confirmed earlier studies on the type and distribution of mucins, and the distribution of reticulin fibres and stainable keratin in the nasopharynx.

A new monoclonal antibody, MA6, which was hoped to be specific or selective for nasopharyngeal carcinoma, did not specifically locate nasopharyngeal epithelial or carcinoma cells by immunohistochemistry. The antigen with which MA6 reacts is widely distributed among both normal and abnormal tissues.

Work was also started on the identification of the Epstein-Barr virus nuclear antigen (EBNA) by modifying the well known anti-complement immunofluorescence test for use on paraffin sections. Many attempts were unsuccessful. The antigen may have been disrupted by the

tissue preparation process, or the primary antiserum may have lacked the high titre and avidity required for immunohistochemistry on paraffin sections.

Using immunohistochemistry of cytokeratins, a pattern of reactivity emerged. Pseudostratified columnar epithelium stained more intensely with AE1/AE3 monoclonal antibody than intermediate epithelium, which in turn stained more intensely than the stratified squamous epithelium. Biopsies showing intraepithelial changes showed a decrease in staining intensity when compared with adjacent normal epithelial counterparts. In particular, patches of squamous metaplasia were easily identifiable among neighbouring normal epithelium. Twenty-seven out of 28 carcinomas tested stained with AE1/AE3. There were no identifiable differences in the staining patterns of the various types of tumour.

Anti-cytokeratin 18 stained only the upper parts of the columnar epithelial cells. Intermediate and stratified squamous epithelia were consistently negative. Areas of hyperplastic columnar epithelium were positive, but all other intraepithelial lesions were unstained. None of the carcinomas stained.

AE1/AE3 could be useful for identification of carcinomas if a differential diagnosis were required between lymphoma, which would be negative, and carcinoma. AE1/AE3 also identified squamous metaplasia, whose significance in the nasopharynx is not yet understood. Squamous metaplasia could be a lesion which is a precursor to malignancy, as it has proved to be in other sites.

Anti-cytokeratin 18 was consistent in that it only identified the simple pseudostratified columnar epithelium. If nasopharyngeal carcinomas carry the same cytokeratin subsets as their precursor cells, then the

lack of reactivity with the carcinomas would indicate that the precursor was not columnar epithelium. However, it is not known whether the expression of cytokeratins changes during the malignant transformation in the nasopharynx. This result, therefore, does not answer the question of histogenesis.

The results of this study indicate that there is selectivity in the histochemical reactions of the normal, the abnormal and the cancerous epithelium of the nasopharynx. Further work will be required to identify the precise differences of these histochemical changes, with a view to aiding histopathological diagnosis and of understanding the histogenetic origin of this common malignant tumour.

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1. INTRODUCTION

The southern Chinese, including those living in Hong Kong are among a number of populations who have a high risk for nasopharyngeal carcinoma (NPC).

Possibly because high incidences of NPC are limited to restricted areas, some aspects of NPC research have been neglected. There have been many studies of the immunology, virology and epidemiology of NPC but the study of the histology of these tumours so far has been limited. This is because there is no universally accepted histological classification and related to this, the histogenesis of the tumours has not been established.

NPC arises from the nasopharyngeal epithelium, but exactly which type of epithelium is not known. The tumour cells have a variety of morphologies and it is because of this heterogeneity there has been a long and confusing debate about their classification. As yet there is no accepted explanation for this variety of appearances. At an international symposium on nasopharyngeal carcinoma held in Japan in 1977[1] five different classifications were promulgated, no two of which were completely interchangeable. Since 1977 new classifications have continued to be submitted to the literature.

A consequence of these problems of histogenesis and classification is that it has been impossible to correlate the results of the many studies that have been made, which discuss histopathology together with other factors such as epidemiology, treatment and prognosis. Some recent studies have indicated that the histological type of tumour may correlate with the patients' outcome. It is therefore essential that a widely accepted histological

classification is established.

This histochemical study was embarked upon with these problems of histogenesis and classification in mind. It was an attempt to find an objective way of defining the different epithelial cell types in normal and abnormal nasopharynx. Following on from this it was hoped that the nature of early intraepithelial changes could be established. Logically this could lead to ways of distinguishing the different types of NPC. Ultimately it may be possible to establish whether there are differences in the histogenesis of the different types of NPC.

The aim was to carry out as much of the work as possible on sections of formalin fixed and paraffin wax embedded tissue, as this is the standard method of tissue preparation for histological study. Tissues thus prepared are routinely stored in histology laboratory files. Retrospective studies can easily be made by cutting new sections off the stored tissue blocks.

Tissues studied included a lymphoblastoid cell line, Raji (whose cells contain one of the Epstein Barr virus antigens, Epstein Barr virus nuclear antigen - EBNA), a NPC tumour xenograft, some post mortem specimens of nasopharynx and a number of nasopharyngeal biopsies showing a range of lesions, from mild intraepithelial changes through to NPC. With the exception of some preparations of Raji cells and some pieces of the xenograft, these tissues were formalin fixed, paraffin processed, and sectioned at 2 or 3 microns.

A number of histochemical investigations were performed on the preparations in order to establish a histochemical profile of the tissues. These included some

well established techniques to identify mucins, reticulin and keratin, and some immunohistochemical techniques using several different antisera.

2. LITERATURE REVIEW

2.1. ANATOMY OF THE NASOPHARYNX

Embryologically the nasopharynx is derived from a recess in the primitive pharynx in the cranial part of the foregut. The recess is formed when the embryo is only a few weeks old.[2]

In the adult the pharynx extends from the base of the skull to the oesophagus and larynx. It is divided into three parts: nasal, oral and laryngeal pharynx. The nasopharynx lies behind the nasal cavities and is the widest part of the pharynx. Because of its bony framework it is always patent under normal conditions.[3,4,5,]

Anteriorly it is continuous with the nasal cavities through the posterior nares which are separated by the posterior edge of the nasal septum. The anterior wall includes the postero-superior surface of the soft palate which forms the incomplete floor of the nasopharynx. The roof of the nasopharynx is formed by the thick layers of the mucous membrane and periosteum on the under surface of the basilar part of the occipital bone. The roof is in continuation with the posterior wall. The posterior wall is formed by the mucous membrane that overlies the prevertebral muscles and fascia, which in turn overlie the anterior arch of the atlas vertebra.[5]

Unlike the other two parts of the pharynx, the nasopharynx has only a respiratory function. During swallowing it is closed off from the oral pharynx by the elevation of the soft palate. At other times there is free communication with the oral pharynx.[6]

The lateral walls of the nasopharynx contain the openings of the auditory or Eustachian tubes whose upper and posterior lips are prominent and cartilaginous and are called tubal elevations or Eustachian cushions.[4]

On either side, folds of the mucous membrane, the salpingopharyngeal folds, descend from the lower edge of each tubal opening to fade out gradually on the pharyngeal wall. Another less distinct pair of ridges of the mucous membrane pass down from the front edge of the tubal openings towards the soft palate, the salpingopalatal folds. Behind the openings of the tubes are deep vertical clefts, the pharyngeal recesses or fossae of Rosenmüller.[5]

The lamina propria of this part of the nasopharynx contains lymphoid tissue, which forms part of Waldeyer's ring of pharyngeal lymphoid tissue. Part of Waldeyer's ring, the nasopharyngeal tonsil or adenoids, is situated in the area where the roof and posterior wall of the nasopharynx merge into one another close to the openings of the Eustachian tubes.[3,5,6]

Lymphatics drain into glands in the retropharyngeal and parapharyngeal recesses which in turn drain into the upper deep cervical chain of the neck. There is free cross over of lymphatics.[2]

2.2. HISTOLOGY OF THE NASOPHARYNX

The nasopharynx is lined by various types of epithelia which are derived from the ectoderm. The epithelium is supported by a connective tissue layer called the lamina propria which contains mixed glands, lymphoid tissue of Waldeyer's ring, and some collections

of lymphocytes without the formation of follicles. Collectively the epithelium and the lamina propria are known as the nasopharyngeal mucosa. A further connective tissue layer called the submucosa connects the mucosa to the underlying structures. The epithelium is separated from the lamina propria by a basal lamina composed of reticulin, collagen and mucopolysaccharide.[7]

Because of its relative inaccessibility, knowledge of the accurate dimensions and of the histology of the normal nasopharyngeal mucosa is incomplete.[8] The only extensive histological study seems to have been made by Ali in 1965.[9] He calculated that the area of the epithelial lining of the adult nasopharynx is approximately 50 sq. cms. Ali identified three types of epithelium: stratified squamous, pseudostratified ciliated columnar and intermediate (or transitional) epithelium.

All three epithelial cell types are found during foetal development.[10] Ciliated cells begin to appear when the foetus measures 29mm crown-rump, and they rapidly develop into the pseudostratified columnar epithelium which is the dominant type at birth. Patches of squamous epithelium begin to appear in 84mm foetuses. The ciliated epithelium begins to be replaced by small patches of squamous epithelium in 84mm foetuses. At full term the roof of the nasopharynx shows mostly pseudostratified columnar ciliated epithelium with patches of non-ciliated columnar, intermediate and squamous epithelium.[11]

In the adult nasopharynx almost 60% of the anterior wall and 80% - 90% of the posterior wall are covered with stratified squamous epithelium. The lateral walls show a pattern of alternating patches of ciliated and squamous epithelia which are separated by islets of intermediate epithelium.

At least 1/3 of the anterior wall abutting the posterior nares and a narrow strip of the roof are lined by pseudostratified ciliated columnar epithelium. Approximately 40% of the anterior wall and 15-20% of the posterior wall are covered with ciliated epithelium. Almost 1/2 the area of the lateral walls is lined by ciliated epithelium distributed in irregular patches alternating with islets of squamous and intermediate epithelia.[9]

The intermediate epithelium forms a wavy junctional zone separating the nasopharynx from the oropharynx.[12] There are numerous islets of intermediate epithelium covering the salpingopharyngeal fold, the pharyngeal recess in the lateral walls and the lining of the pharyngeal tonsils in the posterior wall.

Intermediate epithelium is stratified and 5 or 6 layers thick.[11,13,14] The deepest layers of cells are cuboidal followed by a layer of polyhedral cells and on the surface is a layer of rounded cells. Intercellular bridges are not found by electron microscopy (EM).[10]

The deep layers of stratified squamous epithelium are cuboidal and only the more superficial mature layers are flattened.[7] The cells are non keratinised, although keratohyalin granules can sometimes be seen as basophilic cytoplasmic granules.[9,10] The keratohyalins are antecedents of the keratins of the epidermis.[15]

The pseudostratified ciliated columnar epithelium is composed of several cell types, all of which are in contact with the basal lamina, but not all reach the surface. The cells are ciliated and many of them are mucin producing goblet cells.[7]

2.3. EPIDEMIOLOGY OF NASOPHARYNGEAL CARCINOMA

Most of the neoplasms of the nasopharynx are malignant; only about 10% are benign. Among the malignancies it is the epithelial tumours, that is the nasopharyngeal carcinomas, that are the most common in all races and regions of the world.[8]

2.3.1. GEOGRAPHY

Nasopharyngeal carcinoma occurs commonly in southern Chinese populations, including those living in Hong Kong and in other countries of SE Asia who live a traditional Chinese way of life.[16] Southern Chinese born in Western environments such as Australia, Hawaii and California retain an intermediate risk.[17] NPC accounts for less than 1% of cancers among European races: the corresponding figure among the Chinese in Guangzhou is over 50%[16] and in Hong Kong, 18%.[2] In Guangdong province de Thé reported that NPC comprised 43% of all cancers in males and 16% in females.[18]

In Hong Kong it ranks after bronchus and liver as the third most common malignancy.[19] The male-female ratio is 2.49 - 1.[17] It is the most common cancer among Hong Kong Chinese between the ages of 15 and 34.[19]

In both Hong Kong and Guangdong the incidence rates of NPC for both sexes among the fishing community who live on boats are about two-fold higher than those for the rest of the population.[17,18,20]

An intermediate incidence for NPC has been reported around the Mediterranean in Tunisia,[21] Algeria[22] and Morocco.[23] There is also an intermediate risk in the Rift Valley in Kenya and Uganda and in the Sudan.[16]

Blot et al reported a high incidence among Alaskan natives who are of Eskimo, Indian and Aleutian origin. These people are racially Mongoloid and are widely considered to be of Asian origin.[24] Greenlanders, racially an Eskimo-Caucasian mixture also have an increased risk.[23]

NPC is more common in men than women in all areas. This male preponderance is highest among the Chinese with reported rates of between 2.6 and 4.4 to 1, while in Europe the ratio is less than 2 to 1.[16]

Besides ethnic and geographical features, several other factors have been suggested to be contributory to the development of NPC.

2.3.2. FAMILIAL INCIDENCE AND GENETIC ASSOCIATION

After studying several families of Chinese NPC patients, Ho[25] reported that there is a higher frequency of NPC among close blood relatives of NPC patients than in patients suffering from other types of cancer. 5-6% of the NPC cases seen at the Guangzhou cancer hospital occur in families where two or more cases have been diagnosed.[18] Chan et al reported an increased risk of developing NPC associated with HLA antigens B17, A2 and BW46.[26] There is also a much higher risk if HLA A2 and BW46 occur together. The authors suggested that the posession of such genetic factors can influence the susceptibility of the patient to environmental influences such as exposure to EBV and carcinogens.

2.3.3. EPSTEIN BARR VIRUS

Epstein Barr Virus (EBV) belongs to the gamma herpes group of DNA viruses.[27] It is a ubiquitous virus, being

present in most human populations.[28] EBV was first discovered by Epstein in 1964 when studying electron micrographs of cultured lymphoblastoid cells from an African Burkitt's lymphoma (BL) tumour biopsy.[29,30] Further experiments using indirect immunofluorescence on these cell lines and sera from BL patients confirmed that this was a new virus.[31,32] EBV is also associated with infectious mononucleosis.[33]

Burkitt's lymphoma is a form of undifferentiated lymphoma which commonly occurs in Africa and New Guinea, while being rare in other countries.[34,35] It frequently presents as an osteolytic lesion of the jaw in children 6 - 10 years old.[36,37] It is the most common childhood tumour in Africa. The incidence in endemic areas of Equatorial Africa and New Guinea can be as high as 10/100,000 children[38] The tumour nearly always contains the EBV genome[39] and certain EBV antigens.[40] EBV producing cell lines can be derived from the tumours.[29,30] Patients with African Burkitt's lymphoma have elevated antibody titres to EBV antigens.[41] Most Burkitt's lymphomas in America and Europe do not contain EBV DNA,[42] indicating that the tumour may have several causative factors.

Infectious mononucleosis is a self limiting EBV infection which tends to affect adolescents and young adults living in regions with relatively high socioeconomic conditions, and only affects individuals who lack antibodies to EBV.[43,44] It produces fever, tonsillitis, lymphadenopathy and hepatosplenomegaly. Uncomplicated cases run their course in one to four weeks.[45] Because of the early age of infection with EBV in most parts of the world, very few young adults are susceptible to infectious mononucleosis and it is virtually unknown in most areas.[46]

EBV was first associated with NPC by Old et al[47] who found that serum from NPC patients formed precipitates with extracts from BL cell lines. Later studies confirmed the association[48] and extended it to the demonstration of EBV antibodies in NPC patients' sera by immunofluorescence tests using EBV containing lymphoblastoid cell lines as the source of antigen [49,59]

The role of EBV in the pathogenesis of NPC is not understood, but the association of the two is very strong in cases of NPC from throughout the world.[51] Whether the virus is a passenger or is aetiologically responsible for the development of the tumour is still not conclusively settled.[52] Herpes virus particles have not been definitely observed in EM studies of biopsy material.[53,54] However there is a large volume of indirect evidence to associate EBV with NPC. Both EBV DNA[39,55,56,57,58] and EBV nuclear antigen (EBNA)[59,60] are present in the malignant epithelial cells, while biopsies from other head and neck carcinomas do not contain EBV DNA[39,57]nor do they contain EBNA.[60]

In developing and tropical countries, including those areas with high risk for NPC, most children have been infected by EBV by the age of ten years.[51] NPC patients produce characteristic patterns of serum antibodies to EBV antigens. EBV has an intricate life cycle and different antigens are produced during the various phases of the cycle.[61] In NPC patients, serum antibodies, particularly IgA,[62,63] can be demonstrated in relation to three antigen systems found in the infected cells.[64,65] Viral capsid antigen (VCA) is a structural component of the virus. The early antigens (EA), when investigated in superinfected lymphoblastoid cell lines seem to be composed of at least six distinct polypeptides which are thought to play a role in the transformation of the cell

to an immortalised state.[66]

The Epstein Barr virus nuclear antigens (EBNA) are products synthesised during the quiescent phase. They are localised on the chromosomes and are also thought to play a role in the immortalisation of cells.[67] EBNA is now known to be composed of two distinct polypeptides designated EBNA I and EBNA II.[68] The classical EBNA equates with EBNA I which is a protein mol wt 60-70Kd with an IEP at pH 4.6. EBNA II is more tightly bound to the chromosome and is a basic protein (hence the tight binding), mol wt 60-70Kd with an IEP at pH 9.2.[67,69,70]

NPC has been classified histologically by the WHO into three categories based on the degree of differentiation of the tumour cells.[71] The undifferentiated carcinomas, WHO types II and III, have been associated with EBV for many years. These patients have elevated serological IgA responses to VCA and one of the early antigens (EA-d)[64,72] and DNA hybridisation experiments show the malignant cells also contain EBV DNA.[74] This is regardless of ethnic or geographical origin, as high risk Chinese,[73] medium risk Africans and low risk Caucasian patients[74] with undifferentiated NPC all contain EBV DNA in their tumour cells.

EBV serology of WHO type I (that is tumours showing evidence of squamous differentiation) patients is similar to that of control populations and these tumours have been thought not to have any special association with EBV infection.[72,75]. However recent DNA hybridisation work[58] has indicated that the WHO I tumours do contain EBV DNA, though in much lower genome equivalents than the types II and III generally contain.

The serological response to VCA, EA and EBNA in-

creases with the severity or stage (stage according to Ho)[17] of the disease.[51] The presence of high IgA titres against VCA has been used as the basis of a series of serological surveys undertaken in Wuzhou city, China since 1980, involving at least 20,726 people aged 40 years and over. As citizens reach 40 years they are also recruited to the survey. Those with raised serum IgA titres against VCA were followed up. Altogether 35 cases of NPC were identified, many in the early asymptomatic stages.[76,77]

EBV was thought until recently to be strictly a lymphotropic virus with a very narrow host cell range which would infect only B lymphocytes in vitro, transforming and immortalising them.[78] Monoclonal antibody studies have shown that B lymphocyte cell membranes carry a complement receptor, the C3d receptor of which the CR2 molecule functions as a receptor for EBV.[79] Using the same two monoclonal antibodies against the C3d/EBV receptor of B cells, Young et al[80] demonstrated, by immunofluorescence studies on frozen sections, that the C3d receptor also appears on oral and nasopharyngeal squamous epithelia.

Evidence of EBV replication in exfoliated oropharyngeal cells of infectious mononucleosis patients was reported in 1977[81] but this was not confirmed until recently with further in situ hybridisation experiments on throat washings from infectious mononucleosis patients.[82]

Suggested sites in normal tissues for a lifelong reservoir of EBV, investigated by in situ hybridisation, are parotid gland,[83] and respiratory tract.[84] Additional in situ hybridisation work on various tumours has shown that EBV genomes are present in thymic carcinoma[85] and

salivary gland carcinoma[86] indicating that EBV could also be an aetiological factor in these tumours.

2.3.4. HERBAL MEDICINES

Medicines containing croton oil, derived from the seeds of the shrub Croton tiglium, are used as throat and nasal balms or oils by Chinese.[87] Hirayama and Ito[88] identified a significantly higher relative risk of NPC among patients who had frequently used such herbal medicines.

In a study of 495 Chinese medicinal herbs, 16 herbs including croton oil were able to activate EBV in cultures of latently infected lymphoblastoid cells.[89]

2.3.5. INHALANTS

In a study of Malaysian Chinese, occupational exposure to inhalants such as smoke and dust were significantly associated with NPC.[90] A similar association was found with both smoking and working in poor ventilation.[87] However an additional study which compared risk factors among Hong Kong Chinese and Los Angeles Chinese did not support inhaled carcinogens as major risk factors.[91]

2.3.6. SALTED FISH

Ho reported that one of the traditional foods fed to Southern Chinese children in the weaning and post weaning periods is salted fish added to congee. In a survey of NPC patients, Ho found that salted fish was commonly eaten by boat people from an early age. Boat people are reported to have a particularly high incidence of NPC.[17,18,20] Recent epidemiological studies have shown a

significant association between salted fish intake, particularly during early childhood and NPC.[90,91,92,93]

Salted fish has been found to contain dimethylnitrosamine.[94] In a study in which WA albino rats were fed steamed salted fish, 4/20 rats developed tumours in the nasal and paranasal cavities, but not any of the controls.[95]

2.4. HISTOGENESIS OF NASOPHARYNGEAL CARCINOMA

The histogenesis of the various types of NPC is not understood. The tumours show histological features of epithelial tumours and undoubtedly arise from the epithelium of the nasopharynx.[96] There are three types of epithelium: stratified squamous, intermediate and pseudostratified ciliated columnar.[9] Whether the tumours arise from one or all of these epithelial types is unknown. Several authors agree that the tumours arise from the squamous or pseudostratified columnar epithelium.[13,96,97] In a series of 31 autopsy cases of NPC, Teoh[98] considered that all the tumours arose from the stratified squamous epithelium of the nasopharynx.

In an EM study of 20 specimens Prasad[99] concluded that all types of NPC arise from squamous cells which were either there to start with or after having undergone squamous differentiation. This was on the basis of finding tonofilaments converging onto desmosomes in the malignant cells.

Batsakis et al[10] suggested that different types of tumour arise from normal epithelia that are at different stages of differentiation. The undifferentiated tumours arise from undifferentiated stem cells and the well dif-

ferentiated squamous cell and adenocarcinomas arise from more highly differentiated or determinate epithelial cells. They suggested that intermediate epithelium is particularly susceptible to oncogenic stimuli.

2.5. INTRAEPITHELIAL LESIONS OF THE NASOPHARYNX

Most fully mature, or differentiated cells retain a restricted range of variability, and are able to undergo some changes of phenotype in response to environmental stimuli.[100]

Several distinct non malignant lesions which may be attributed to environmental stimuli, can be identified in the nasopharyngeal epithelium. These may or may not progress to malignancies.

2.5.1. METAPLASIA

This is the conversion in post natal life of one differentiated cell type to another. True metaplasias arise in epithelial renewal tissues when there is chronic tissue regeneration caused by trauma, infection or abnormal hormonal stimulation. The presence of metaplasias in a situation of continuous cellular renewal indicates that the transformation occurs at the level of the stem cells as these are the only cells that survive the tissue indefinitely. The existence of small foci of metaplasia suggests a monoclonal origin of the lesions. For a monoclonal lesion to grow to macroscopic size the transformed tissue must compete with regenerating normal tissue. Changes from glandular to squamous epithelium are thought to give greater resistance to mechanical and chemical irritation.[101]

This squamous metaplasia is apparently reversible on the removal of the irritating stimulus. However there are instances where the cancers probably arise from a cell within a patch of metaplasia and more closely resemble the metaplasia than the normal parent tissue. The fairly uncommon squamous cell carcinomas and adenocarcinomas of the bladder probably arise within areas of metaplasia. Squamous cell carcinoma of the bronchus is also believed to arise from patches of squamous metaplasia. Here squamous metaplasia is attributed to smoking, although smoking probably has other carcinogenic effects which may contribute to the development of squamous cell carcinoma.[101]

The occurrence of metaplasia in areas of chronic tissue regeneration is well established. Implications in terms of cancer risk may be small in some tissues but are probably considerable in others.[101] Areas of squamous metaplasia are identifiable in the nasopharyngeal epithelium. The implications of these metaplasias are unknown.

2.5.2. HYPERPLASIA

An increase in the number of cells in an adult tissue is termed hyperplasia. Hyperplasia may be divided into two different but overlapping types: (a) physiological and (b) pathological or neoplastic. Physiological hyperplasia is a normal reaction of tissue to environmental stimuli such as irritation and infection, while neoplastic hyperplasias are caused in part at least by an inherited abnormality within the cells. Physiological hyperplasia occurs because of an increase in the proportion of replicating cells which continue to divide instead of undergoing differentiation and eventual death. On removal of the stimulus, physiological hyperplasia is revers-

ible.[100] Areas of hyperplasia are seen in all types of nasopharyngeal epithelium.

2.5.3. KOILOCYTES

Cells of the epithelium that are undergoing koilocytic change are characterised by a prominent nucleolus, a perinuclear halo and vacuolated cytoplasm. They can occur in all epithelial layers and have been reported in 'hairy' leucoplakia lesions of the tongue and gingiva of human immunodeficiency virus (HIV) infected patients, in plantar and common warts of the skin and in condylomatous lesions of the cervix uteri. Several investigations using DNA hybridisation and immunohistochemistry have associated these lesions with both EBV[102,103] and human papillomavirus (HPV)[104,105,106] infections. Cells of similar appearance are seen in nasopharyngeal epithelium, although they have not been associated with viral infection.

Condylomatous lesions of the cervix have a high rate of spontaneous regression and it has been suggested that many lesions classified as cervical intraepithelial neoplasia grade I (see below) are in fact HPV induced condylomata.[104]

2.5.4. INTRAEPITHELIAL NEOPLASIA

This expression was first introduced with regard to the precursor lesions of squamous cell carcinoma of the cervix, as cervical intraepithelial neoplasia (CIN). The implication of the term was that regardless of morphological appearance, all precancerous intraepithelial abnormalities of the cervix belong to the same family of lesions. The lesions are graded from I to III, with CIN grade I corresponding to the morphologically lesser le-

sions and grade III to the classical severe dysplasia or carcinoma in situ. All the lesions have common nuclear abnormalities such as nuclear enlargement, hyperchromasia and an increased number of mitoses, many occurring in abnormal locations and possibly showing abnormal mitotic figures. The lesions differ from one another by the degree of epithelial disarrangement and cell maturation.

In CIN grade I lesions the epithelial stratification is well preserved and the cells have abundant well differentiated cytoplasm. CIN grade III shows a marked disarrangement of the epithelial structure and scanty or keratinised cytoplasm. The CIN grade II lesions show nuclear and cytoplasmic features intermediate between the two groups.[107]

It has been suggested that a comparable sequence of events takes place in the nasopharynx, and that the genesis of cancer in the nasopharynx is preceded by recognisable intraepithelial lesions.[108]

Nasopharyngeal intraepithelial neoplasia (NPIN) type I shows isolated atypical cells in the lower two-thirds of the epithelium or numerous undifferentiated cells in the lower third. NPIN type II has many undifferentiated cells in the lower two thirds, while NPIN grade III shows numerous undifferentiated cells in the full thickness of the epithelium.[108]

2.6. HISTOLOGICAL CLASSIFICATION

NPC is the most common type of malignancy of the nasopharynx, particularly in countries with a high incidence of the disease. In Singapore, for example, 98.7% of the malignancies of the NP are carcinomas, whereas in

the UK 70.2% and in Japan 79.2% of nasopharyngeal malignancies are carcinomas. Adenocarcinomas, lymphomas and other cancers make up the remainder.[109]

NPC can arise from any part of the nasopharynx. The most common sites are the lateral walls, particularly the fossa of Rosenmüller and eustachian cushion, followed by the roof. Less common sites are the posterior and and anterior walls.[2,10,13] The lateral walls are areas where the intermediate epithelium is most common.[10]

For some time there has been debate over the histological subclassification of NPC. This is partly because the histogenesis of the tumours has not been clarified, partly because of its occurrence in a variety of histological forms,[8,10] partly due to subjective interpretations by pathologists and partly because some early ultrastructural studies claimed evidence of squamous differentiation in all NPCs.[110] Only those cells whose cytoplasmic filaments converge into desmosomes are now considered to have undergone squamous differentiation.[10]

Malignant tumours of the NP were first described by Trotter in 1911 as 'endotheliomas'. In 1921 Schminke and Regaud independently described tumours of the NP which had an admixture of lymphocytes as 'lymphoepitheliomas.'[98] Since then many reports and classifications have appeared which use these or different names leading to confusion, particularly because the term 'lymphoepithelioma' could misleadingly indicate that the tumours are of mixed lymphoid and epithelial origin.[111]

Teoh[98] in a study of 31 autopsies considered the lymphocytes of the so-called lymphoepitheliomas to be in-

cidental components and not an integral part of the tumours. Yeh[13] found that 87% of a series of 1000 malignancies of the nasopharynx were epidermoid carcinomas. He identified seven different types based on the degree of differentiation and the cell morphology. Although he used it in his classification, Yeh doubted the existence of lymphoepithelioma as a separate entity. He suggested that it was a transitional cell carcinoma infiltrating the pre existing lymphoid tissue of the NP. In this study he failed to equate tumour type with prognosis.

Liang et al[97] classified their series of 50 autopsies and 500 biopsies into three groups and six types according to the degree of differentiation and the morphology of the cells. The well differentiated group include cylindrical cell (adenocarcinoma) and squamous cell carcinoma grades I and II. The poorly differentiated tumours are large cell, fusiform cell and squamous cell carcinoma grade III. Liang et al's poorly differentiated tumour is their pleomorphic cell carcinoma. The authors equated these groups with modes of spread. The undifferentiated tumours tending to show both cranial base invasion and distant metastases while the highly differentiated tumours tended not to invade the base of the skull nor to metastasise.

Classifications continued to be submitted to the literature and during an international symposium on nasopharyngeal carcinoma held in Kyoto, Japan in 1977,[1] five different classifications were presented, no two of which were completely interchangeable.[96,112,113,114] Descriptive terms used included undifferentiated carcinoma, undifferentiated carcinoma of nasopharyngeal type, nonkeratinising carcinoma and anaplastic carcinoma.

The diversity of terms used in these and other

classifications, and the absence of a uniform histological classification has not allowed correlation of the different studies that have appeared in the literature which discuss histopathology together with other aspects of NPC such as epidemiology, treatment and prognosis.[10,52,109] In an attempt to rectify the confusion, in 1978 the WHO proposed a classification into three types: keratinising squamous cell, non keratinising carcinoma and undifferentiated carcinoma.[71] See Table 1.

Since the introduction of the WHO classification, modifications to the scheme and new classifications have continued to be submitted to the literature,[111,115,116] each claiming to be indicative of prognosis. Table 2 compares these recent classifications with the WHO classification.

All of the schemes retain the category of squamous cell carcinoma (SCC) as a distinct entity. The French scheme combines the non-keratinising and the keratinising tumours of WHO together in a single group called undifferentiated carcinoma of nasopharyngeal type (UCNT). They justified this division into two categories on the basis of EBV serology; patients with SCC had low titres of EBV related antibodies while patients with UCNT had elevated titres. They also claimed good correlations of age, ethnic origin and survival rates after treatment (no details supplied), and suggested that SCC and UCNT are two distinct diseases of the nasopharynx.[117]

The Taiwan scheme introduces three groups, besides SCC, based on cell morphology: spindle cell (SC), mixed cell (MIX) and round cell (RC). Each of these is further subdivided according to the degree of differentiation. Types A are undifferentiated and types B are well differentiated.[116]

TABLE 1. WHO CLASSIFICATION OF NASOPHARYNGEAL CARCINOMAS[71]

TYPE I	Squamous cell carcinoma (or keratinising squamous cell carcinoma (SCC). Showing definite evidence of squamous differentiation with the presence of intercellular bridges and keratinisation over most of its extent. It may be graded as well, moderately or poorly differentiated.
TYPE II	Non-keratinising carcinoma (NK). Showing evidence of differentiation with a maturation sequence that results in cells in which squamous differentiation is not evident on light microscopy. The tumour cells have fairly well defined cell margins and show an arrangement that is stratified or pavemented. A plexiform pattern is common. There is no evidence of mucin production, or of glandular differentiation.
TYPE III	Undifferentiated carcinoma (UC). The tumour cells have oval or round vesicular nuclei and prominent nucleoli. The cell margins are indistinct and the tumour exhibits a syncitial rather than a pavemented appearance. Spindle shaped tumour cells are arranged in irregular and moderately well defined masses and/or strands of loosely connected cells in a lymphoid stroma. These cells do not produce mucin.

TABLE 2. HISTOLOGICAL CLASSIFICATION OF NASOPHARYNGEAL CARCINOMA

WHO scheme[71]	French scheme[111]	Cologne scheme[115]	Taiwan scheme[116]
Keratinising squamous cell carcinoma, Type I (SCC)		Keratinising squamous cell carcinoma, Type I (SCC)	Keratinising squamous cell carcinoma
	Squamous cell carcinoma (Keratinising or not)		Spindle cell carcinoma Type A undifferentiated Type B well differentiated
Non-keratinising carcinoma, Type II (NKC)		Non keratinising carcinoma, Type IIa, without lymphoid infiltration	Mixed cell carcinoma Type A undifferentiated Type B well differentiated
		Type IIb, with lymphoid infiltration	
Undifferentiated carcinoma, Type III (UC)	Undifferentiated carcinoma of nasopharyngeal type (UCNT)	Undifferentiated carcinoma, Type IIIa without lymphoid infiltration	Round cell carcinoma Type A undifferentiated Type B well differentiated
		Type IIIb with lymphoid infiltration	

The authors claim that this classification correlates closely with patient outcome. KS has the worst prognosis with 5 year survival rate of 21.1%, SC 5 year survival rate of 41% with RC and MIX at 51.2% and 54.1% respectively. Among the types A and B, the undifferentiated tumours (A) have a much less favourable outcome than the well differentiated (B) types.

The Cologne scheme[115] essentially follows the WHO system, but it subdivides the types II and III into IIa and IIIa (without lymphoid infiltration) and IIb and IIIb (with lymphoid infiltration). Patients with SCC have the worst prognosis, followed by NK and UC. Those with a heavy lymphoid infiltrate have a significantly better outcome than those without.

Patients with tumours classified by the WHO scheme had five year survival rates of 11% for SCC, 27.6% for NK and 30.1% for UC. Those with a heavy lymphocyte infiltrate, perhaps reflecting a more effective host response, had a better prognosis over three years but not over five years. The three and five year survival rates were better for females (55.3% and 42.3%) than for males (26.0% and 19.5%) respectively.[109]

2.7. HISTOCHEMISTRY

Few histochemical studies of the nasopharynx have been undertaken, and histochemistry has been of little use as an aid to the diagnosis of NPC.

In Ali's[9] comprehensive study of the histology of the NP he reported that keratohyalin was demonstrated (no method given) occasionally in intermediate epithelium and more frequently in the squamous epithelium, particularly

in individuals over 50 years of age. He also identified melanin pigment using, Schmorls reaction, in the basal cells of the epithelium. Melanin was more common in Indians and Malays than in Chinese. Numerous mast cells were demonstrated in the lamina propria using Dominici's (metachromatic?) stain.

In the nasopharyngeal mucosa, mucin secretion is confined largely to goblet cells in the ciliated columnar epithelium with occasional intermediate cells also containing cytoplasmic mucin. In a thorough histochemical investigation using twelve techniques including PAS, alcian blue, mucicarmine and toluidine blue, Tock & Tan[118] established that practically all the mucins of the nasopharynx are either sialidase resistant sialomucins or sulphated mucins. The submucosal glands also secrete sialidase resistant sialomucins and sulphated mucins. A few glands produce neutral mucins only. The gland ducts produce mucins that are similar to those of the surface epithelium. Non keratinising and undifferentiated (and presumably squamous cell) carcinomas do not produce mucins.[71]

Glycogen, demonstrated by Best's carmine, was reported to be present in the clear cells of an undifferentiated tumour, and in liver metastases of a second autopsy case of NPC.[98] Liang et al[97] noted that well differentiated tumours were often encapsulated by argyrophilic reticulin fibres while the poorly differentiated and undifferentiated tumours were usually without encapsulation.

Lennert et al[119] recommended using Giemsa stain to help diagnose undifferentiated carcinoma of nasopharyngeal type (UCNT). The grey-blue tumour cells can be easily distinguished from dark blue lymphocytes and

plasma cells.

UCNT cells are moderately positive for acid phosphatase and weakly positive for non specific esterase and chloroacetate esterase. Use of these enzyme methods facilitates the distinction between UCNT, particularly its metastases, and Hodgkins disease and lymphosarcoma, which are negative for all three enzymes.[117,119,120]

In a light and electron microscope study of a series of 434 NPC biopsies Prathap et al[121] reported the presence of intra- and extracellular amyloid in 12% of the specimens. They demonstrated it for light microscopy with congo red, crystal violet and thioflavine T and confirmed it with EM studies. The tumours were classified by the WHO system and amyloid was present in all three histological types. It was most common in non keratinising carcinomas with 22% of those examined showing amyloid.

2.8. IMMUNOHISTOCHEMISTRY

Considerably more use has been made of immunohistochemistry in the diagnosis of NPC, than of histochemistry.

Epstein Barr virus nuclear antigen (EBNA) was demonstrated in touch smears made from fresh NPC biopsies[59,60,117] by the anti complement immunofluorescence (ACIF) test.[122] The ACIF has been modified to an anti complement immunoenzyme (ACIE) method using peroxidase instead of a fluorescent dye as a label.[123] These techniques use polyvalent human antisera to EBNA as the primary antibody.

Various other antigens have been investigated by immunohistochemistry as potential markers for NPC, using both polyclonal and monoclonal antibodies. These include the epithelial cell markers epithelial membrane antigen (EMA), carcinoembryonic antigen (CEA) and keratin (or cytokeratin).[124,125,126,127,128,129,130,131,132]

EMA is a large glycoprotein which is present on most secretory epithelia and secreted by many carcinomas.[133,134] It was originally demonstrated with antisera raised against defatted human cream.[135] It is widely distributed in human carcinomas,[134,136,137] and occasionally found in lymphomas and plasmacytomas.[138] Reports vary as to the value of EMA as a tumour marker. Sloane et al[137] found it to be a more efficient tumour marker than cytokeratin. Both Pinkus et al[138] and Thomas and Battifora[139] found both monoclonal and polyclonal antibodies to cytokeratin to be more effective than antibodies to EMA for the characterisation of epithelial derived neoplasms.

CEA is an epithelial membrane antigen which was first identified in human foetal intestine and adult colon cancer by Gold and Freedman in 1965[140,141] and thought by them to be a specific marker for colorectal cancer. Immunohistochemical studies have shown that CEA is a product of normal as well as malignant colonic cells.[142,143,144] It has also been localised in tumours of the small bowel, testis, breast, thyroid, lung and cervix.[133,145,146,147,148] It is typically absent from sarcomas and lymphomas.[149]

Gusterson et al[127] found that their polyclonal antibodies to EMA and CEA were less reliable for the recognition of NPC than their polyclonal antibody to keratin. EMA was weakly positive while CEA was positive in only

two out of 38 carcinomas tested by Bosq et al.[130,132] All the carcinomas were negative for the lymphoid marker leucocyte common antigen and for the melanoma associated antigen.[130,132]

S-100 protein is an acidic, calcium binding protein initially identified in the brain and called S-100 because of its solubility in 100% ammonium sulphate.[150] It was at first used as a marker for glial cells of the central nervous system and Schwann cells of the peripheral nervous system,[151] but was later found to be more widely distributed. Melanocytes, chondrocytes, myoepithelial cells and breast ducts were among the cells found to express S-100. Their malignant counterparts also stain with anti S-100, and this led Kahn et al to suggest that the presence of S-100 in some tumours can be used to confirm their histogenesis.[150]

S-100 protein is also present in a number of cells with dendritic features which have been identified as both Langerhans cells (or T-zone histiocytes) and interdigitating reticulum cells by immunohistochemistry and ultrastructural studies. These cells have been identified in a number of pathological specimens, including NPC.[152,153,154] They are thought to have an immunological role, possibly in the presentation of antigen to T-lymphocytes.[154] Because of their reactivity with antibodies to S-100 protein it has been suggested that interdigitating reticulum cells are related to cells of the macrophage/monocyte system.[155] In Bosq et al's study using a panel of monoclonal antibodies against a number of tumours, anti S-100 protein gave weak staining in about half the undifferentiated NPCs tested. The type of cells stained was not described.[130]

Wick et al evaluated reactivity of a number of

tumours, including 36 NPCs, with antibodies to placental-like alkaline phosphatase (PLAP), EMA and cytokeratin. The NPCs were uniformly negative with PLAP and positive with EMA and cytokeratin in contrast to the 37 germ cell tumours which were positive with PLAP and variable with EMA and cytokeratin antibodies.[156]

From these and other studies, cytokeratin emerges the most useful marker for the histological diagnosis of NPC that has been found to date.

2.8.1. CYTOKERATINS

The cytokeratins are one of the five classes of intermediate filaments that make up part of the filamentous cytoskeleton of vertebrate cells.[157] Intermediate filaments are the 10nm components of the cytoskeleton, as distinguished from the 6nm actin filaments and the 25nm microtubules.[158] They are composed of a family of five types of proteins which can be distinguished biochemically and immunologically.[159] The cytokeratins are characteristic of epithelial cells; vimentin occurs in mesenchymally derived cells; desmin filaments are typical of muscle cells; neurofilaments occur in neurons and glial filaments are found in astrocytes.[160,161]

The cytokeratin filaments are composed of a complex family of at least seventeen different polypeptides (See table 3). They were catalogued by Moll[159] who distinguished them by their isoelectric points which range from 5.1 to 7.8 and by their molecular weights which range from 40 to 67Kd.

The seventeen members of the cytokeratin family are divided into two subfamilies, the type A and type B subfamilies (or types I and II):

TABLE 3. CLASSES OF CYTOKERATINS[159,162]

CYTOKERATIN SUBFAMILY					
TYPE A (ACIDIC)			TYPE B (BASIC)		
mol wt Kd	pI	Moll's CK no	mol wt Kd	pI	Moll's CK no
56.5	5.3	10	67	7.8	1
55	4.9	12	65.5	7.8	2
54	5.1	13	64	7.5	3
50	5.3	14	59	7.3	4
50	4.9	15	58	7.4	5
48	5.1	16	56	7.8	6
46	5.1	17	54	6.0	7
45	5.7	18	52	6.1	8
40	5.2	19			

The type A subfamily comprises acidic polypeptides of pI 4.5 - 5.5, mol wt 40 - 57Kd, that is cytokeratins (CK) 10 to 19 of Moll's catalogue.[159]

The type B subfamily comprises basic polypeptides of pI 5.5 - 7.5, mol wt 52 - 68Kd, CK 1 - 8 of Moll's catalogue (See table 3).

Each cytokeratin 10nm intermediate filament contains at least one polypeptide member from each subfamily. Pairs of cytokeratins consisting of one specific member of the type A subfamily and one specific member of the type B subfamily seem to be consistently expressed in different types of epithelial cells.[162] Certain pairs are only found in simple epithelia (45Kd type A or CK 18 and 52Kd type B or CK 8) while others are found in stratified epithelia (50Kd type A or CK 14 and 58Kd type B or CK 5).[158,162,163]

Besides those cytokeratin polypeptides that are constant to the 10nm intermediate filaments of each cell type, subsets of two to ten cytokeratins are expressed in any given epithelium. The detailed composition of each subset is heterogeneous and can vary depending upon cell type, degree of differentiation and disease state.[159,162,164,165] It is not yet understood how the inclusion of different cytokeratin polypeptides in the assembly of the 10nm filaments alters the resulting properties of these filaments.[165]

It should therefore be possible to identify and classify epithelial cells by the composition of their cytokeratin subset. Most epithelial tissues can be classified as being simple or stratified, but a few do not fall into these categories. These include the transitional epithelium of bladder and tracheal pseudo-

stratified ciliated columnar epithelium. It is possible that the intermediate and the pseudostratified ciliated columnar epithelium of the nasopharynx fall into a similar category. Generally cytokeratins expressed by these unusual epithelia represent a combination of stratified and simple types.[166,162]

Epithelial derived malignancies (carcinomas) continue to produce cytokeratins,[124,167,168] but comparisons between the carcinomas and their normal cellular counterparts have shown that the pattern of cytokeratins produced by the abnormal cells is sometimes quite different.[167] Some tumours, for example adenocarcinoma of colon, have been found to express cytokeratin patterns nearly identical with those produced by their specific cells of origin. Others, such as breast cancer and adenocarcinoma of lung, have been found to express only a subset of the cytokeratins produced in the corresponding normal tissues.[165,167]

Antibodies to intermediate filament proteins have been used extensively as diagnostic markers for various human neoplasms.[168,169] These antibodies can distinguish between carcinomas, lymphomas, melanomas and sarcomas and can be helpful in diagnosing undifferentiated tumours.[170] Antibodies to cytokeratin have been shown to recognise tumours of epithelial cell origin.[138,157,170,171,172,173]

The earliest study involving the identification of cytokeratin in NPC was by Schlegel et al in 1980.[124] They briefly reported that formalin fixed and paraffin processed NPC tissues gave strongly positive antibody reactivity with a rabbit anti human keratin antiserum by a peroxidase-anti-peroxidase technique. This was followed by further studies using polyclonal antisera.[125,126,127] All the NPCs tested in these reports gave positive stain-

ing. More recent studies have used monoclonal antibodies to cytokeratin[128,129,130,131,132,139] in which the large majority of carcinomas tested stained positively.

2.8.2. OTHER MARKERS

MA6 is a murine IgM monoclonal antibody[174] which reacts with an antigen found on NPC cells. The immunogen for MA6 was an immunoprecipitate obtained by counterimmunoelectrophoresis of serum from an NPC patient and an extract of lysed Raji cells, a Burkitt lymphoma cell line.

MA6 reacts with an antigen which has been identified as a 55Kd glycoprotein, or a group thereof,[175] which is referred to as B-lymphocyte carcinoma cross-reacting antigen (BLCa). BLCa has been demonstrated on B lymphocytes, on carcinoma cells including NPC[174] and on some nasopharyngeal epithelial cells.[176]

In immunofluorescence tests, MA6 reacts with a number of B lymphocyte cell lines. Immunoperoxidase staining of several formalin fixed and paraffin processed tumour tissues gave positive staining in carcinomas.[174] Immunocytochemistry of impression smears of NPC biopsies resulted in positive staining in tumour cells, some lymphoid cells and some epithelial cells.[176]

Suspensions of exfoliated NPC cells were subjected to a direct binding assay using ^{125}I labelled MA6.[177] Six out of nine patients tested gave positive binding. Results of Western blotting experiments on extracts of NPC biopsies correlated closely with immunocytochemistry of impression smears taken from the same specimens before extraction.[176]

2.9. TECHNIQUES USED IN IMMUNOHISTOCHEMISTRY

Immunohistochemistry has been defined as the identification of a tissue constituent in situ by means of a specific antigen-antibody reaction tagged by a visible label.[178] This essentially simple principle is dependent upon the fact that low molecular weight compounds such as fluorescein, enzymes and biotin can be attached to an antibody without interfering with its function as an antibody.[179] The various techniques used have descended from an immunofluorescence method of Coons[180,181] for the demonstration of antigens in tissue culture cells. In the early techniques the specific antibody itself was labelled (direct method),[180] and later the more sensitive and versatile indirect method was introduced.[182] Fluorescein isocyanate was replaced by fluorescein isothiocyanate[183] which is simpler to conjugate to antibodies and it has remained the fluorescent label of choice. It yields an intense apple green fluorescence on irradiation in the near UV (excitation at 490nm, emission at 520nm).[178] Fluorescence methods are still used extensively because of their speed and simplicity.

Disadvantages inherent in the fluorescence methods are that they require a fluorescence microscope, background details cannot be seen clearly and the preparations are not permanent because the fluorescent labels cannot withstand dehydration so aqueous mounting media must be used. Several labels have been used as alternatives to fluorescent dyes. For light microscopy the enzyme labels have been the most successful. Horseradish peroxidase[184] is most commonly used. The enzyme labels must be developed histochemically to produce coloured insoluble end products at the site of reaction.

2.9.1. ANTICOMPLEMENT IMMUNOFLUORESCENCE TEST

The anticomplement immunofluorescence (ACIF) test[122] was developed to identify EBV antigens in human lymphoblastoid cell lines carrying the Epstein Barr virus and applied to cell smears from two Burkitt's lymphoma biopsies. The test revealed a complement fixing antigen localised in the nucleus of the cultured cells and in the nuclei of the Burkitt's lymphoma cells. The Burkitt's lymphoma cells showed a more coarsely granular fluorescence than the fine granules of the cultured cells. The antigen was designated EBV-associated nuclear antigen (EBNA).

Smears of the cell lines were prepared either in the cytocentrifuge or by spreading a concentrated suspension of washed cells on microscope slides, air drying and fixing in cold acetone. Sera obtained from Burkitt's lymphoma and NPC patients were tested for the EBV capsid antigen and early antigens. Those with raised titres were used as test sera. Serum with no detectable EBV antibodies was used as a source of complement.

The smears were incubated with the test serum (which had first been diluted optimally and incubated at 56°C for 30 min. to inactivate complement) together with complement at 37°C in a humid chamber. After washing, the second step was an incubation with an optimally diluted fluorescein conjugated rabbit anti human complement. Slides were examined with a fluorescence microscope.

The ACIF test was subsequently applied to touch smears of NPC biopsies which had been air dried and rapidly fixed in cold methanol-acetone.[59] Results showed granular fluorescence in the nuclei of the malignant cells. The presence of EBNA positive cells correlated

with raised serum titres of antibodies to EBV antigens.[60]

Zeng et al modified the ACIF test[123,185] to an immunoenzymatic method. Their preparations were cold acetone fixed smears of cells from the NP, which had been collected by negative pressure suction.[185,186] The first step of the procedure was the same as the ACIF. The second step of the ACIF was replaced by an incubation with a horseradish peroxidase conjugated rabbit anti human complement. The peroxidase was visualised with diaminobenzidine-hydrogen peroxide which produces a brown deposit at the site of reaction. Results showed brown nuclear staining which was comparable with the fluorescence of the ACIF test. EBNA was detected in malignant cells, hyperplastic cells and ciliated columnar epithelial cells. The test was weakly positive in squamous epithelial cells. EBV negative sera gave negative results. The authors claimed that the ACIE was more sensitive than the ACIF test and it was easier to see the morphology of the cells. In addition it obviated the need for an expensive fluorescence microscope. The ACIF and ACIE techniques can only be applied to smears and frozen sections. They have not yet been successfully used on paraffin processed sections.[117]

2.9.2. IMMUNOENZYME METHODS

Since the introduction of enzyme labels for immunohistochemistry and the widespread application of the techniques to paraffin processed tissues, there has been a proliferation of work in the identification of cell markers for tumour diagnosis.[135,149,187,188]

Horseradish peroxidase,[184] E. coli alkaline phosphatase[189] and Aspergillus niger glucose oxidase[190] have been used as antibody labels. Endogenous peroxidase

activity in granulocytes and erythrocytes must be inhibited prior to immunostaining with methanol-hydrogen peroxide[191] or periodate oxidation.[192] Endogenous alkaline phosphatase must be blocked with levimasole[193] or periodate oxidation. Glucose oxidase occurs exclusively in plants and requires no blocking step. Horseradish peroxidase has become the most commonly used enzyme label. Alkaline phosphatase has been used particularly in double immunolabelling techniques[189] and is now becoming popular as a single antigen label.

2.9.3. INDIRECT

More sensitive than the direct method,[180] the section is first incubated in primary antibody. After washing in buffer, the second, labelled antibody is applied to the section. The second antibody is from another species, raised to the IgG of the animal donating the first antibody.[182] Visualisation of the label reveals the site of the primary antigen. The early methods utilised fluorescent labels. Enzyme labels were introduced later.[194] The increase in sensitivity is because each primary antibody binds several molecules of the second antibody.[179]

2.9.4. UNLABELLED ANTIBODY-ENZYME METHOD

The peroxidase-anti-peroxidase (PAP) technique[195] involves the use of a stable complex of three peroxidase molecules and two anti peroxidase antibody molecules which acts as a third layer antigen. The first layer is (for example) a rabbit anti antigen. the second layer a goat anti rabbit IgG and the third layer a rabbit PAP complex. The PAP is more sensitive again than the indirect method because of the increased amount of label. The primary antibody can be highly diluted, thus reducing

the amount of background staining, a problem particularly of polyclonal antibodies. A similar complex using the alkaline phosphatase-anti-alkaline phosphatase complex (APAAP) is also in use, particularly for double immunoenzymatic labelling[189]

2.9.5. AVIDIN-BIOTIN

Avidin is an egg white glycoprotein (approx. 68 Kd) that has a high affinity for the small molecule vitamin H or biotin (244Daltons). Avidin is a tetramer and has four binding sites for biotin. By covalently coupling biotin to an enzyme molecule the enzyme acquires the ability to bind avidin. Covalent coupling of biotin to antibody gives the antibody the ability to bind avidin.[196] These properties have been utilised in the avidin-biotin-peroxidase methods.[197,198] The first step is the primary antibody; the second a biotinylated antibody raised in another species against the IgG of the animal donating the primary antibody; the third step is an avidin-biotin-peroxidase complex in which three out of the four available binding sites on the avidin molecule are occupied by biotin-peroxidase, the fourth site is free to bind with the biotin of the second layer antibody. The technique is considerably more sensitive than the PAP method enabling high dilutions of primary antibody to be used. It facilitates the demonstration of antigens which have been partially lost during fixation and processing. The increased sensitivity may be due to multiple binding which brings more enzyme to the site,[179] or because the ABC complex is comparatively small so that penetration of reagents to the tissue is easier than the with the large PAP complex.[199,200]

2.9.6. STREPTAVIDIN-BIOTIN

Despite the high affinity of avidin for biotin, problems of non specific binding have been reported and attributed to the stickiness of the carbohydrate moiety of avidin. In addition the pI of avidin is 10 which may cause ionic binding with anionic tissues, resulting in non specific binding.

Streptavidin, which is isolated from cultures of Streptomyces avidinii, has similar properties to avidin: it is a tetramer, molecular weight around 60kD and it binds biotin with an extremely high affinity. On the other hand it is non glycosylated and has a neutral pI. These two properties make streptavidin superior to avidin as a reagent for the detection of biotinylated molecules.[201] It is now used extensively in immunohistochemistry for both light and electron microscopy.[202]

2.9.7. ENZYME SUBSTRATES

Probably the most commonly used substrate for peroxidase development is diaminobenzidine tetrahydrochloride with hydrogen peroxide (DAB-H_2O_2).[203] DAB gives a brown insoluble deposit at the site of reaction which is insoluble in alcohol and xylene. Solvent based mounting media can be used to make permanent preparations with good resolution. Several alternative substrates are available but they tend to be less satisfactory than DAB. The coloured deposits of 3-amino-9-ethyl carbazole (red)[204] and 4-chloro-1-naphthol (blue)[205] are alcohol and xylene soluble so aqueous mounting media must be used. Preparations therefore are not permanent and resolution is not as good as with synthetic mounting media. Hanker-Yates reagent (purple)[206] deteriorates on storage and tends to be capricious in use. However these

alternative substrates are useful in double immunoenzymatic methods when contrasting coloured deposits are required.

A substrate for alkaline phosphatase labelled reagents is naphthol-AS-MX phosphate plus fast red or fast blue which give red or blue deposits at the reaction site. The deposits are alcohol soluble so an aqueous mounting medium is required.[207]

Glucose oxidase can be visualised with phenazine methosulphate plus a tetrazolium salt such as p-nitro blue tetrazolium chloride.[190] The end product is resistant to dehydration and clearing.

2.9.8. PROTEOLYTIC ENZYMES

Preliminary proteolytic enzyme digestion enhances immunohistochemical staining with a variety of antigens in paraffin sections, especially if the tissue is formalin fixed, for example immunoglobulins, alpha-1-anti trypsin and keratin.[208,209,210] A number of enzymes have been evaluated to optimise immunoreactivity of various tissue antigens, including pronase, papain, pepsin and trypsin. Several studies have indicated that trypsin is the most suitable[210,211] as digestion is more easily controlled and morphology is better preserved.

The type of trypsin, the concentration and the digestion time are important in obtaining good results, and must be optimised for each laboratory. In an immunohistochemical study of cytokeratin of a variety of formalin fixed and paraffin processed carcinomas using both polyclonal and monoclonal antibodies, Pinkus et al[210] obtained good results with type II porcine trypsin (Sigma).[212]

2.9.9. POLYCLONAL AND MONOCLONAL ANTIBODIES

Immunohistochemical procedures rely on the availability of antibodies that will react in a specific way with the tissue antigen. Until 1975 all antibodies were produced by injecting a purified preparation of the antigen into an animal, thus inducing production of specific antibodies against the antigen. After a suitable time interval the animal is bled and the serum collected contains the soluble antibodies. Animals used include rabbits, goats, swine, sheep, horses and guinea pigs. However, an immunohistochemical technique is only as reliable as the specificity of the antibodies used in the system and the specificity of the primary antibody is crucial.[213] These polyclonal antisera are heterogeneous and contain antibody subpopulations that differ in region specificity and avidity. They may contain contaminating antibodies either induced by previous infections/immunisations or evoked by unknown contaminants in the immunogen preparation. Such antisera may not be suitable for critical immunocytochemistry as the techniques will indiscriminately detect all antibodies that bind to a tissue section regardless of specificity.[214]

The unwanted antibodies are best removed by affinity purification in which the antigen under investigation is coupled to a solid support such as cyanogen bromide activated Sepharose beads. The desired antibody population will bind while the contaminating antibodies are washed away. The specific antibodies are then eluted. The problem with affinity purification is that high avidity antibodies, which are ideal for immunocytochemistry, are difficult to elute completely.[216] However, affinity purified antibodies are used extensively in immunocytochemistry.

In 1975 Kohler and Milstein devised an ingenious method of producing pure and reproducible antibody in unlimited quantities.[216] Mice of an certain strain, for example, BALB/c, are immunised and when the antibody is being produced the mice are killed. Dissociated plasma cells from their spleens, the source of the antibodies, are fused with cultured myeloma cells from the same strain of mice.

These hybrid cells retain the properties of both parent cells. that is, of antibody production and of continuous growth in culture. The hybrid cell population will produce as many assorted antibodies as the original immunised mouse. Pure antibodies are obtained by continuously dividing the cultures and testing the supernatants for antibodies. Only cultures producing the wanted antibody are kept and these are further divided and tested until clones derived from a single cell and producing specific antibody are obtained. The culture medium contains the antibody to the original antigen, which is of homogeneous specificity and constant affinity.[178]

A lengthy technique, but as long as the culture is kept alive, an indefinite supply of monospecific antibody is available. The advent of monoclonal antibody technology thus circumvents the difficulties of lack of specificity of polyclonal antibodies. The field of immunohistochemistry has benefited enormously from the application of monoclonal antibodies. They react specifically with the tissue antigen, giving the minimum of background staining.[178]

It should be remembered, though, that a monoclonal antibody against a single well defined antigenic determinant does not necessarily mark a single family of

cells. The same (or similar) determinants may be found on cells that are developmentally unrelated.[213]

3. MATERIALS AND METHODS

3. MATERIALS

3.1. CELL LINES

Two cell lines were obtained from Dr DP Huang, Department of Morbid Anatomy, Prince of Wales Hospital, Shatin, Hong Kong.

3.1.1. RAJI

This is a lymphoblastoid cell line of African Burkitt's lymphoma origin which has B cell features.[217] It carries EBV DNA[218] and the EBV-determined nuclear antigen (EBNA)[122] is demonstrable. Raji cells were used as a positive control for the modified anticomplement immunofluorescence test (ACIF). The original ACIF test is used to identify EBNA in cultured cells as well as in clinical material.[59,122]

3.1.2. BJA-B

A lymphoblastoid B-cell line from an African Burkitt-like lymphoma which shows no evidence of the EBV genome when tested by DNA hybridisation and immunofluorescence for EBNA, EA and VCA.[219] BJA-B cells were used as a negative control in the modified ACIF test.

These two cell lines were prepared in two ways: smears and paraffin processed blocks.

3.1.3. SMEARS

A concentrated suspension of pH 7.2 phosphate buffered saline (PBS) washed cells was spread on clean glass slides, air dried and fixed in equal volumes of acetone and methanol at -10°C, or in 10% buffered formalin or in periodate-lysine-paraformaldehyde (PLP) for 3 min (see appendix 1). Smears were stored in a sealed box at -20°C until used. PLP has been used successfully as a fixative for immunoelectron microscopy and for immunohistochemistry of nervous tissue.[220,221]

3.1.4. PARAFFIN PROCESSED BLOCKS

PBS washed cells were pelleted by centrifugation and fixed in equal volumes of -10°C acetone-methanol or in 10% buffered formalin or in periodate-lysine-paraformaldehyde for 1 hour. The fixed cell pellets were processed to paraffin wax by a standard 18 hour schedule. They were sectioned at 2 or 3 microns, mounted on chrome alum-gelatine coated slides[222] (see appendix 1) to ensure adhesion of the section, and baked in a 56°C oven overnight. They were stored at room temperature until used.

3.2. XENOGRAFTS

The undifferentiated form of NPC has proved difficult to grow in tissue culture. Consequently tumour xenografts, grown in athymic nude mice have been used as a means of studying the tumour under controlled conditions in a constant and reproducible form.

Two xenografts were obtained from Mrs Margaret Lui, Institute of Radiation and Oncology, Queen Elizabeth Hospital, Kowloon, Hong Kong, to test the ACIF method.

One, 1216, contained the EBV genome. The other, 1252, was negative for EBV. The tumour tissues were halved and fixed in either -10°C acetone-methanol or 10% buffered formalin for six hours. The fixed tumour pieces were processed by a standard 18 hour schedule. They were sectioned at 2-3 microns, mounted on chrome alum-gelatine coated slides (see appendix 1) and baked in a 56°C oven overnight. They were stored at room temperature in a closed box until used.

3.3. BIOPSY OF NASOPHARYNGEAL CARCINOMA

A single fresh-frozen biopsy was obtained from Mr Stephen Lo, Department of Morbid Anatomy, Prince of Wales Hospital, Shatin (PWH). It was mounted on a cryostat chuck and sectioned at 5 microns. The sections were picked up on microscope slides, air dried and fixed in -10°C acetone-methanol for 10 min. The sections were wrapped in aluminium foil and stored at -20°C until used.

3.4. AUTOPSY SPECIMENS

Four autopsy specimens of nasopharynx were obtained from Mr Stephen Cheung, Department of Morbid Anatomy, PWH. They had been removed by the rapid method of Szanto[223] and comprised the nasopharynx together with part of the sphenoid bone and sphenoid sinus.

3.4.1. A86-76: male, aged 81 years. Cause of death: carcinoma of oesophageal-gastric junction.
Gross examination: received fixed in buffered formalin, a diamond shaped piece of nasopharyngeal mucosa approximately 10 * 10 cm attached at one end to the sphenoid bone. The openings of the eustachian tubes,the tubal elevations and the fossa of Rosenmüller could be seen. The sphenoid bone was

dissected away, and the entire mucosa cut up for histological examination into 30 blocks (see figure 1) which were processed to paraffin wax by a standard 18 hour schedule.

3.4.2. A87-368: male aged 54 years. Cause of death: disseminated lymphoma.
Gross examination: received fresh, a rectangle of nasopharyngeal mucosa approximately 7 * 5 cm attached at one end to the sphenoid bone. The opening of the eustachian tube, the tubal elevation and the fossa of Rosenmüller could be seen on the left side. The right side was incomplete. Six representative blocks were taken from the mucosa in the area of the eustachian tube and fossa of Rosenmüller (see figure 2). They were fixed in buffered formalin for 24 hours and processed to paraffin wax by a standard 18 hour schedule.

3.4.3. A87-371: male aged 76 years. Cause of death: myocardial infarction.
Gross examination: received fresh, a rectangular piece of nasopharyngeal mucosa approximately 5 * 5 cm, attached to the sphenoid bone. The opening of the eustachian tube, the tubal elevation and the fossa of Rosenmüller could be seen on the left side. The right side was incomplete. Five representative blocks were taken from the area of eustachian tube and the the fossa of Rosenmüller (see figure 3). They were fixed in buffered formalin for 24 hours and processed to paraffin wax by a standard 18 hour schedule.

3.4.4. A87-376: male aged 59 years. Cause of death: carcinoma of lower oesophagus with secondaries of the lung, liver and stomach.

Gross examination: received fresh, a diamond shaped piece of nasopharyngeal mucosa approximately 6 * 4.5 cm, attached to the sphenoid bone. The opening of the eustachian tube, the tubal elevation and the fossa of Rosenmüller could be seen on the left side. The right side was incomplete. Two depressions 5mm long were noted in the anterior right mucosa. Nine blocks were taken from the mucosa in the area of the eustachian tube and the fossa of Rosenmüller (see figure 4). They were fixed in buffered formalin for 24 hours and processed to paraffin wax by a standard 18 hour schedule.

All paraffin blocks were sectioned at 3-4 microns, mounted on chrome alum-gelatine coated slides and baked in a 56°C oven overnight. The slides were stored in a closed box at room temperature until used.

3.5. BIOPSIES OF NASOPHARYNX

3.5.1. INTRAEPITHELIAL LESIONS

46 paraffin processed biopsies of nasopharynx were obtained from the histology files of the Department of Morbid Anatomy, PWH. They had already been diagnosed and demonstrated the following intraepithelial changes:

hyperplasia	N = 4
metaplasia	N = 24
koilocytes	N = 14
intraepithelial neoplasia	N = 10

They were sectioned at 2-3 microns, mounted on chrome alum-gelatine coated slides and baked in a 56°C oven overnight. They were stored at room temperature in a

closed box until used.

3.5.2. CARCINOMAS

31 paraffin processed biopsies were obtained from the histology files of the Department of Morbid Anatomy, PWH. They had already been diagnosed and classified by the WHO system.[71] They showed the following lesions:

keratinising squamous cell carcinoma (SCC)	N = 1
undifferentiated carcinoma (UC)	N = 27
including some variants:	
large cell	N = 2
spindle cell	N = 1
small cell	N = 1

They were sectioned at 2-3 microns, mounted on chrome alum-gelatine coated slides and baked in a 56°C oven overnight. They were stored at room temperature in a closed box until used.

3. METHODS

3.6. MODIFIED ANTICOMPLEMENT IMMUNOFLUORESCENCE TEST

This procedure is detailed in appendix 3. This was performed on the smears and paraffin sections of the Raji and BJA-B cells, on frozen sections from the fresh biopsy of NPC and on the paraffin sections of the xenografts 1216 and 1252. The rationale of the modification was to apply the sensitive streptavidin-biotin technique to the well established ACIF test.

The ACIF test has only been used on smears of cul-

tured cells, impression smears from tumour biopsies and frozen sections.[117] It has not been used successfully on paraffin processed tissues. Reasons for this lack of success are not understood. It could be that the antigen, EBNA, is partially or completely destroyed in the fixation and processing procedure. It was thought that if it were only partially destroyed, as is the case with many antigens, a highly sensitive method such as the streptavidin-biotin technique would localise small quantities of residual antigen.

In the ACIF test,[59,122] smears from lymphoblastoid cell lines, impression smears from Burkitt's lymphoma biopsies or impression smears from NPC biopsies are prepared, air dried and fixed in cold acetone or acetone-methanol. Primary antisera are obtained from Burkitt's lymphoma or NPC patients with raised titres to EBV antigens VCA, EA and EBNA, as determined by immunofluorescence on Raji cell smears.[224] Non-specific nuclear staining is excluded by testing the sera on a T-cell line which is free of EBV DNA and EBNA. Negative control serum is anti-EBNA negative. Serum with no detectable EBV antibodies is used as a source of complement.

In the first step the smears are incubated with the primary antiserum (which is first diluted optimally and incubated at 56°C for 30 min to inactivate complement) together with complement in a humid chamber at 37°C. After washing, the second step is an incubation with an optimally diluted fluorescein conjugated rabbit anti human complement. Slides are examined with a fluorescence microscope.

When applied to cold acetone-methanol fixed impression smears of NPC biopsies, the procedure has been changed slightly by incubating in the primary antiserum

and the complement in two separate steps.[59] In both techniques granular fluorescence is seen in the nuclei of the positive cells, although the fluorescence in the cultured cells is finer than that of the smears prepared from biopsies.

In the modified ACIF method, step one was an incubation in the primary antiserum, which was serum from NPC patient 10290. The serum had been screened for EBV and antinuclear antibodies and complement had been inactivated as described above. Step two was to apply biotinylated anti human IgG, and step three a streptavidin-biotin-complex which was conjugated to preoxidase. The peroxidase was visualised with DAB-H_2O_2 (see appendix 1) which gave a brown insoluble deposit at the site of reaction. Table 4 compares the ACIF and this modification. Details of the modification are given in appendix 3. The test was repeated using the primary antiserum at a variety of dilutions: 1:10, 1:20, 1:40, 1:80, 1:100, 1:200 and 1:400 to establish an optimum dilution. The primary antiserum was omitted in negative controls.

TABLE 4. MODIFIED ACIF TEST TO DEMONSTRATE EBNA

	ACIF[59,122]	Modification
1.	Incubate with optimally diluted anti EBNA serum obtained from NPC patients.	
2.	Incubate with human complement, from EBV negative serum.	Incubate with biotinylated rabbit anti human IgG
3.	Incubate with fluorescein conjugated rabbit anti complement.	Incubate with streptavidin-biotin-peroxidase complex.
4.	-	Visualise peroxidase with DAB-H_2O_2

3.7. HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

The following techniques were performed on paraffin sections from the autopsy cases and on the NP biopsies. Details of the techniques are given in appendix 2.

3.7.1. HAEMATOXYLIN AND EOSIN

All sections were stained with Mayer's haematoxylin and eosin.

3.7.2. SOUTHGATE'S MUCICARMINE [225]

This is one of the early empirical techniques for the demonstration of mucins. It uses a complex solution which is specific for mucins, although neutral mucins and some strongly sulphated mucins stain weakly or not at all. Other acid mucins stain strongly. The staining mechanism is not clearly understood but it is probable that aluminium salts in the solution form a carmine-mordant complex which bonds with the negatively charged acid mucins.[229,227]

Southgate's mucicarmine was used to stain mucin in all the autopsy specimens and the malignant biopsies of nasopharynx. The method is given in appendix 2.

3.7.3. ALCIAN BLUE[228]

A copper phthalocyanine dye used in the textile industry and first used as a biological stain by Steedman in 1950.[15] In 1957 the original alcian blue 8GS was replaced by the new alcian blue 8GX. New improved staining procedures were worked out by Mowry.[229] Alcian blue contains positively charged groups which will form ionic bonds with some tissue polyanions. These polyanions are the sulphate and carboxyl radicals af the acid mucins. The staining of acid mucins by alcian blue is considered to be specific. By varying the pH of the solution more information can be obtained regarding the types of acid mucins that are present. At pH 0.2 only the highly sulphated mucins are ionised and react. At pH 1.0 both weakly and strongly sulphated mucins react, while at pH 2.5 most acid mucins will stain. It must be noted, however, that with these techniques, it is not always possible to obtain clear cut demarcation of the different types of mucin.[15,226,227]

Sections from all autopsy specimens, and from the malignant biopsies of the nasopharynx were stained with alcian blue at pH 1.0 and pH 2.5. Details of the methods are in appendix 2.

3.7.4. PHLOXINE-TARTRAZINE[230]

A trichrome technique which was originally described as a general histological stain which could be used for the demonstration of inclusion bodies. It also demonstrates fibrin, Paneth cells and keratin, when it is stainable. It relies on overstaining with red phloxine, followed by progressive substitution of the phloxine with tartrazine in 2-ethoxyethanol which acts as a yellow counterstain.

All sections from the autopsy specimens and the malignant biopsies were stained with phloxine-tartrazine for the demonstration of stainable keratin in the epithelia. The method is given in appendix 2.

3.7.5. GORDON AND SWEETS' SILVER IMPREGNATION FOR RETICULIN FIBRES[231]

Reticulin fibres (collagen type III) are fine branching fibres which are normally found connected to stronger and coarser collagen fibres. They make up a supporting framework in soft tissues such as liver, spleen and lymph node, and are associated together with polysaccharides with basement membranes and basal laminae. Reticulin fibres are best demonstrated with one of the many silver impregnation techniques which give good contrast. These methods are all modifications of the Bielschowsky techniques for nerve fibres in which silver from silver oxides is selectively deposited on the fibres. It is then converted into visible reduced black

silver. Most reticulin techniques are preceded by a permanganate oxidation step which probably prevents argyrophilia of nerve fibres. The final step is to remove any remaining unreduced silver by treating with sodium thiosulphate which prevents background precipitation, due to light reduction[226,227]

Gordon and Sweets' technique is a popular and reliable method which gives sharp black impregnation of the reticulin fibres, collagen is orange-brown while the background is clear. It was used on all autopsy specimens and on all the malignant biopsies of nasopharynx, for the demonstration of the basal laminae which separate the epithelia from the underlying lamina propria. The detailed method is given in appendix 2.

3.8. IMMUNOHISTOCHEMICAL TECHNIQUES

Sections were tested with three monoclonal antibody preparations: AE1/AE3 (Hybritech),[232] Cytokeratin 18 (Amersham)[233] and MA6 which was a gift from Professor M H Ng, Department of Microbiology, University of Hong Kong.

Optimum dilutions and incubation times had to be established for each antibody, and for the linking reagents.

3.8.1. DILUTIONS AND INCUBATION TIMES OF SECOND AND THIRD IMMUNOREAGENTS

The second layer antibody for all three primary monoclonal antibodies was a biotinylated rabbit anti mouse immunoglobulins (Dakopatts).[234] This antibody had been raised against a mouse serum pool, affinity purified and biotinylated (product information). The third im-

munoreagent was a streptavidin-biotin-peroxidase complex (Amersham).[233] Using AE1/AE3, at a dilution of 1:150 for 30 min as the primary antibody, the biotinylated rabbit anti mouse immunoglobulins (Igs) and the streptavidin-biotin-peroxidase complex were set up in a series of chequerboard titrations. Paraffin sections of buffered formalin fixed carcinoma of oesophagus were used as control material.

The first involved incubating with biotinylated rabbit anti mouse Igs at dilutions of 1:200, 1:250, 1:300, 1:400 and 1:500 and in streptavidin-biotin-peroxidase complex at dilutions of 1:100, 1:200, 1:300, 1:400 and 1:500 for 30 min each. All dilutions were prepared in 0.05M TBS pH 7.6 and all incubations were done in a humid chamber at room temperature.

When the optimum dilutions were established a second set of slides were stained, varying the staining times of the second and third immunoreagents. Times of 30 min, 1 hour, 1 hour 30 min and 2 hours were used for both reagents.

3.8.2. AE1/AE3 ANTIBODIES (Hybritech)

This is a mixture of two monoclonal antibodies which were prepared against SDS-denatured human epidermal callus keratins[163] by the hybridoma technique.[216] Culture media from the hybridoma lines are used as a source of antibodies.

AE1 is an IgG2a antibody which recognises a number of cytokeratins of the acidic A subfamily.

Mol wt (Kd)	Moll's CK no[159]
40	19
48	16
50 (pI 5.3 & 4.9)	15,14
54	13
56.5	10

AE3 is an IgG2a antibody which recognises several cytokeratins of the basic B subfamily.

Mol wt (Kd)	Moll's CK no[159]
52	8
54	7
56	6
58	5
59	4
64	3
65-67	2-1

The AE1/AE3 antibody pool is supplied by the manufacturer as a 20:1 mixture of AE1 to AE3. This combination is said to recognise all known epithelial cytokeratins[162] and has no known cross reactivities with other cytoskeletal filaments.[163,164]

AE1/AE3 was used in this investigation on paraffin sections of all autopsy and biopsy specimens of nasopharynx. A streptavidin-biotin-peroxidase (Amersham)[233] followed by DAB/H_2O_2 (see appendix 1) visualisation procedure was used to locate the antigens.

Demonstration of cytokeratin in buffered formalin fixed and paraffin processed tissue is enhanced with preliminary treatment of sections with trypsin in order to expose antigenically active binding sites. However, the time of trypsinisation must be optimised for each laboratory.[210] Similarly, optimum dilutions and incubation times of the primary antibody must be established. A block of buffered formalin fixed, paraffin processed carcinoma of oesophagus was used as control material.

To establish optimum trypsinisation time, sections were treated with 0.1% trypsin with 0.1% calcium chloride in 0.05M TBS, pH 7.8 at 37°C for 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105 and 120 min. See appendix 1 for details of trypsin preparation. The remainder of the schedule was standardised as follows: AE1/AE3 at 1:200 overnight; biotinylated rabbit anti mouse Igs at 1:250 for 30 min; streptavidin-biotin-peroxidase complex at 1:200 for 30 min.

For optimum dilution of AE1/AE3 antibody, trypsinised sections were incubated at room temp in a humid chamber at dilutions of 1:100, 1:200, 1:300 and 1:400 for 30 min, 1 hour, 2 hours and overnight. The remainder of the schedule was standardised at trypsin 1 hour; biotinylated rabbit anti mouse at 1:250 for 30 min; streptavidin-biotin-peroxidaes complex at 1:200 for 30 min. Dilutions were prepared in 0.05M TBS pH 7.6. A humid chamber was used for all incubations. Non immune mouse serum replaced the primary antibodies in negative controls. Details of procedures are given in appendix 3. When optimum conditions had been established, sections from autopsies and all biopsies were immunostained with AE1/AE3.

3.8.3. ANTI-CYTOKERATIN 18 (Amersham)[233]

This monoclonal antibody was raised against the kidney epithelium cell line, PtK1. Cells were lysed in a nonionic detergent (1% Nonidet P-40 in PBS pH 7.5). The washed, centrifuged, insoluble material was sonicated and used as the immunogen.[235] Monoclonal antibodies were prepared by the hybridoma technique.[216] Among a number of antibodies produced by this fusion, the IgG2a antibody, LE65, was found by immunofluorescence to react with simple epithelia, and by 2-dimensional 'Western' blotting to react with a 45Kd, pI 5.7 cytokeratin, or cytokeratin 18 by Moll's classification.[159]

Anti cytokeratin 18 is supplied by Amersham[233] as a concentrated cell culture supernatant. Optimum conditions for immunostaining were established by testing a section of buffered formalin fixed, paraffin processed renal biopsy. Trypsinisation (see appendix 1) times were optimised by treating sections for 0, 10, 20, 30, 40, 50 and 60 min in trypsin, followed by immunostaining with anti cytokeratin 18 diluted to 1:15 overnight. The antibody was diluted in PBS (0.15M NaCl plus 0.05M phosphate buffer pH 7.2) containing 1% bovine serum albumin (supplier's recommendation) at dilutions of 1:5, 1:10, 1:15 and 1:20 for two hours and overnight. Again all incubations were carried out in a humid chamber. Anti CK 18 was replaced by non immune mouse serum in negative controls. Details of procedures are given in appendix 3. When optimum conditions had been established, sections from autopsies and all biopsies were immunostained with anti CK 18.

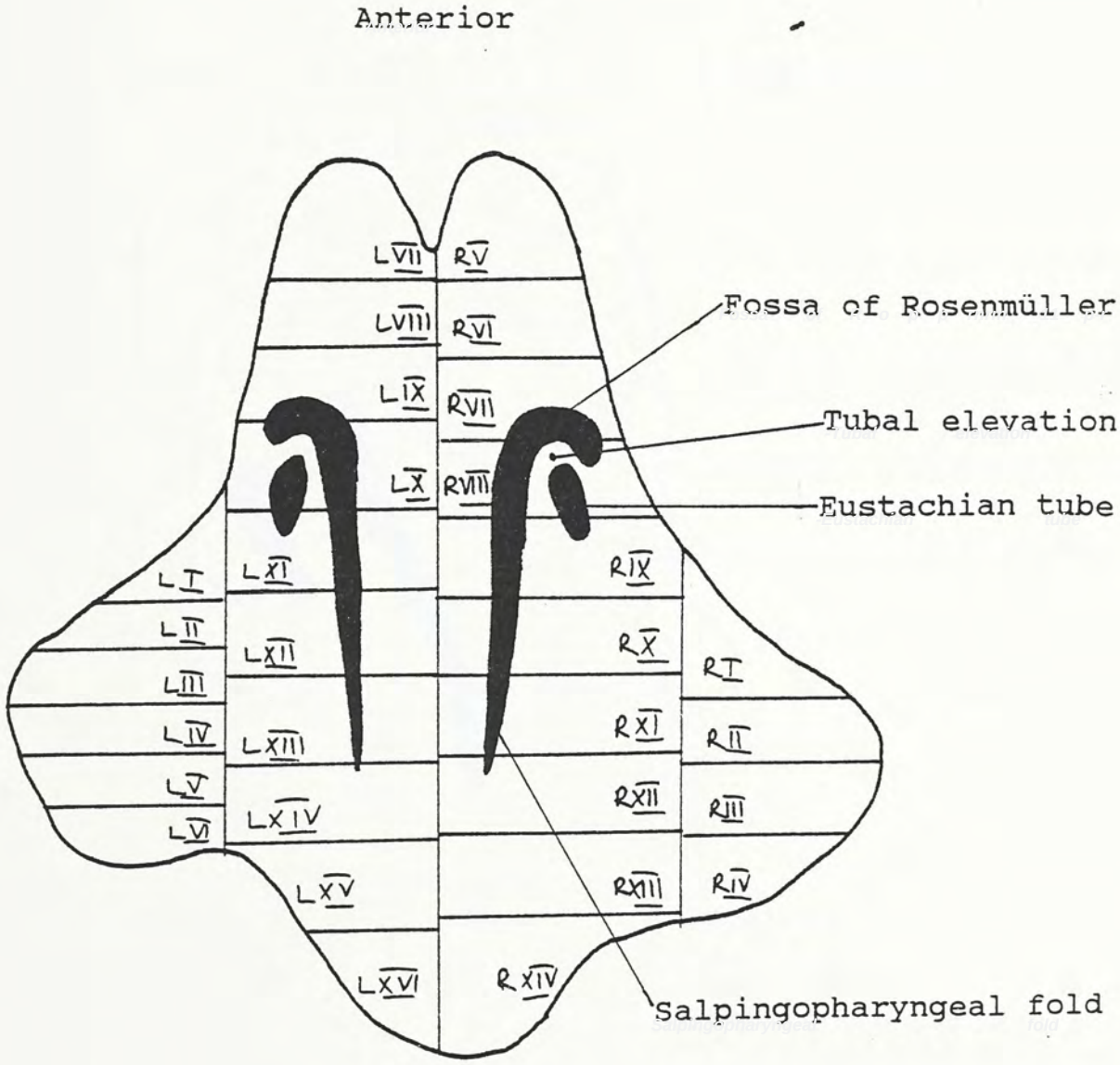
3.8.4. MA6 ANTIBODY

This antibody was a gift from Professor M H Ng, Department of Microbiology, University of Hong Kong. See section 2.8.2. for details of its production. It was supplied as a cell culture supernatant.

Optimum dilution was established by testing on sections of buffered formalin fixed, paraffin processed nasopharynx from autopsy. The antibody was diluted in 0.05M TBS pH 7.6 to 1:100, 1:150, 1:200, 1:250 and 1:300, and incubated for 30 min, 1 hour, 2 hours and overnight at room temp in a humid chamber. Non immune mouse serum replaced MA6 in negative controls.

To try and eliminate non specific background staining, sections were immunostained after trypsinisation (see appendix 1) for 10, 20, 30, 40, 50 or 60 min. When the optimum procedure was finalised, sections from autopsies and the malignant biopsies were immunostained with MA6 antibody. Details of the procedure are given in appendix 3.

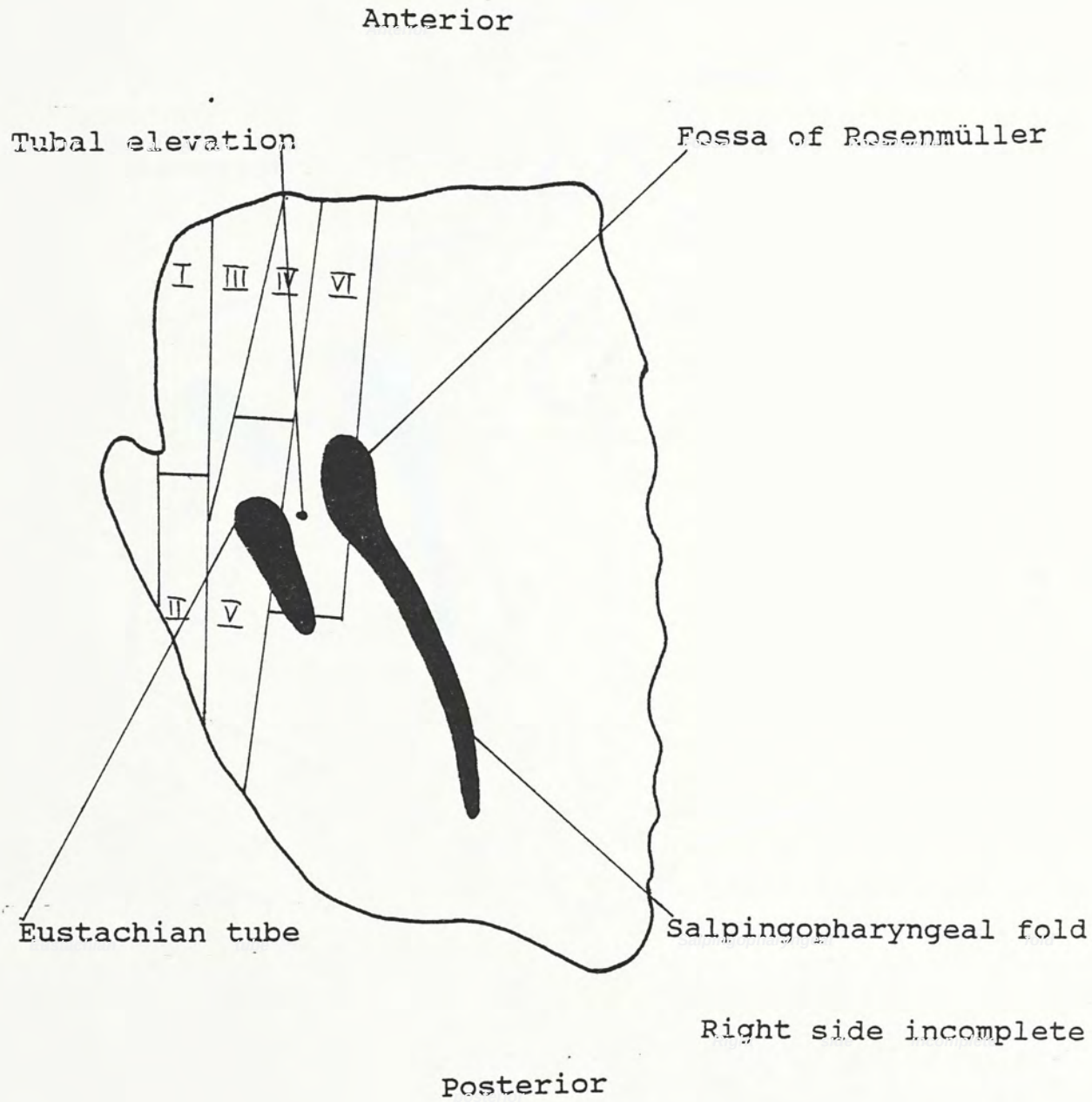
Figure 1. STRUCTURE AND DISSECTION OF NASOPHARYNX A86-76



KEY

LI - LXVI
RI - RXIV = Blocks taken for histology

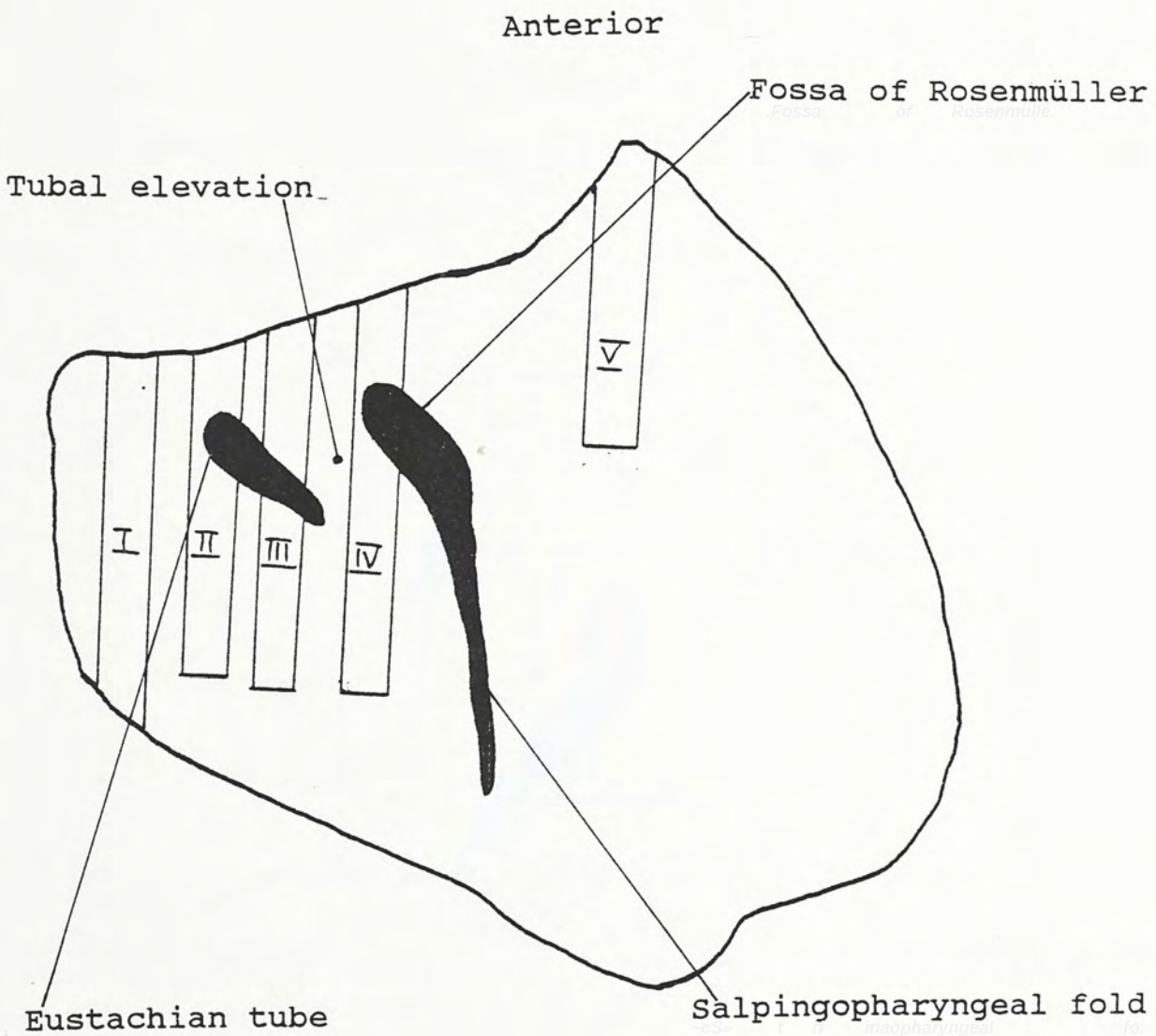
Figure 2. STRUCTURE AND DISSECTION OF NASOPHARYNX A87-368



KEY

I - VI = Blocks taken for histology

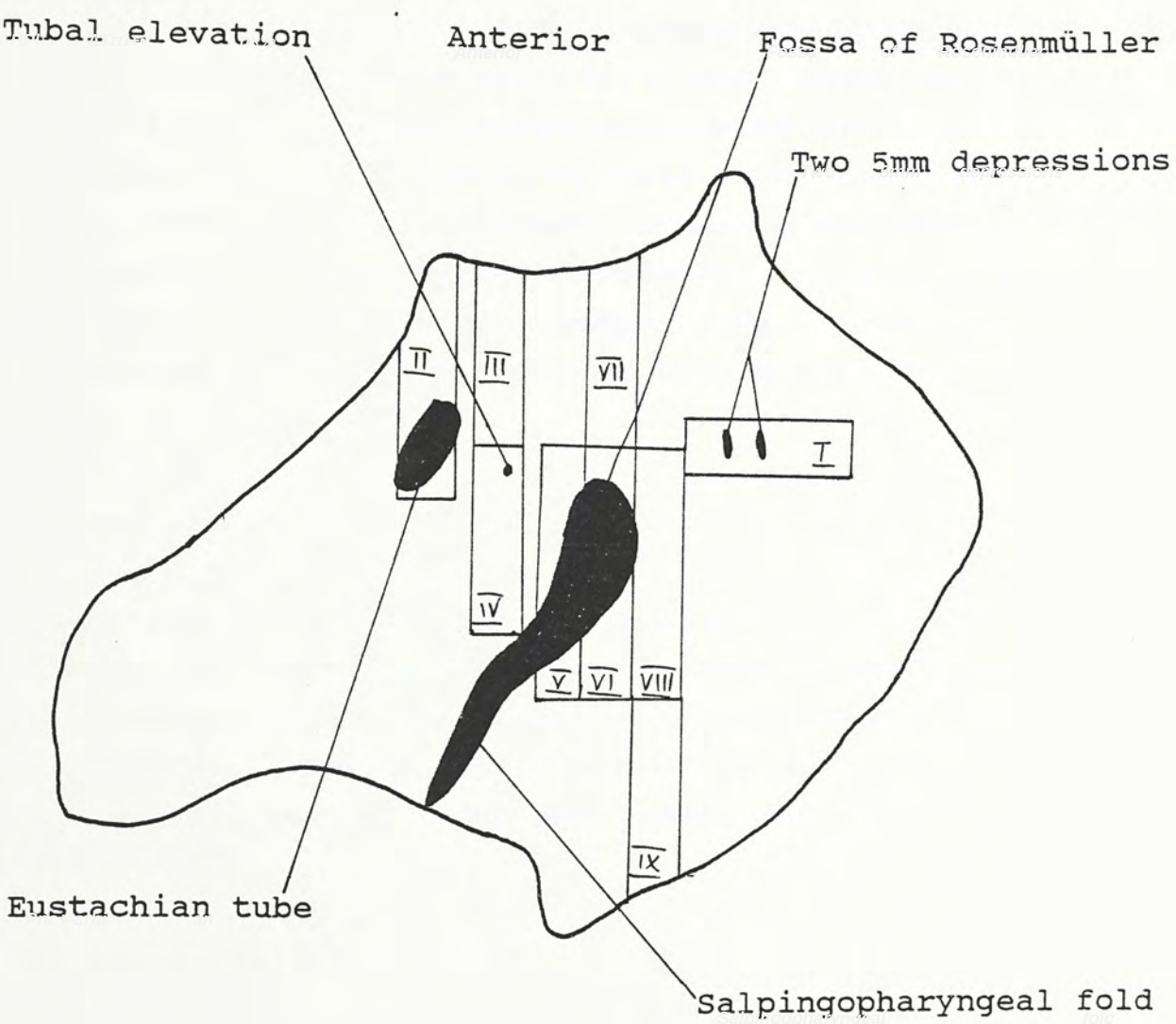
Figure 3. STRUCTURE AND DISSECTION OF NASOPHARYNX A87-371



KEY

I - V = Blocks taken for histology

Figure 4. STRUCTURE AND DISSECTION OF NASOPHARYNX A87-376



KEY

I - IX = Blocks taken for histology

4. RESULTS

4.1. ACIF MODIFICATION

4.1.1. CELL SMEARS

Smears of Raji cells showed brown granular staining in the nuclei of cells fixed in acetone-methanol or in buffered formalin. PLP-fixed cells were negative. The pattern of staining was comparable with the granular fluorescence seen in the ACIF test. Optimum nuclear staining, with minimal cytoplasmic staining was seen with the primary antiserum, 10290, diluted to 1:200 or 1:400. Acetone-methanol gave better morphological preservation than buffered formalin. BJA-B cell smears were uniformly negative.

4.1.2.CELL SECTIONS

Sections of Raji cells showed brown granular staining in the nuclei of cells fixed in acetone-methanol or in buffered formalin. See figure 9. PLP-fixed cells were again negative. Optimal dilution of primary antiserum 10290 was again between 1:200 and 1:400. BJA-B cells were negative.

4.1.3 XENOGRAFTS

Variations of dilutions and incubation times gave no staining of the paraffin sections from both xenografts.

4.1.4. BIOPSY OF NPC

The modified ACIF gave no nuclear staining, but many lymphocytes and plasma cells had brown cytoplasmic staining.

4.2. OPTIMISING IMMUNOHISTOCHEMISTRY PROTOCOLS

4.2.1. DILUTIONS AND INCUBATION TIMES OF SECOND AND THIRD IMMUNOREAGENTS

Chequerboard titrations for optimum dilutions of biotinylated rabbit anti mouse Igs (B-RAM) and streptavidin-biotin-peroxidase complex (Stra-B-C). Optimum staining was achieved when B-RAM was diluted to 1:250 and Stra-B-C to between 1:200 and 1:400. Lower dilutions resulted in background staining while higher dilutions gave weak staining. See table 5.

A similar titration for incubation times indicated that prolonging incubation times over 30 min did not significantly improve demonstration of cytokeratin. Incubation times of 30 min were therefore adopted for both reagents.

4.2.2. TRYPSIN INCUBATION TIME WITH AE1/AE3 ANTIBODIES

Trypsinisation. Optimum staining was achieved if sections were treated with trypsin for between 1 hour and 1 hour 30 min prior to immunostaining. Times in excess of 1 hour tended to cause sections to become detached from the slides, so a time of 1 hour was adopted.

4.2.3. DILUTION AND INCUBATION TIME OF AE1/AE3

AE1/AE3. A final chequerboard titration resulted in overnight incubation with AE1/AE3 diluted to between 1:200 and 1:400 giving best results. See table 6.

4.2.4. TRYPSIN INCUBATION TIME WITH ANTI-CYTOKERATIN 18

Using the renal biopsy as control material, 20 min trypsinisation gave the best results with heaviest stain-

ing in the distal convoluted tubules.

4.2.5. DILUTION AND INCUBATION TIME OF CYTOKERATIN 18

The chequerboard titration resulted in optimum staining at dilutions of 1:5, 1:10 or 1:15 overnight. Because of the expense of anti CK 18, a 1:15 dilution was adopted for all tests. Results are summarised in table 7.

4.2.6. DILUTION, INCUBATION TIME AND TRYPSIN INCUBATION WITH MA6 ANTIBODY

The conditions which gave the least background staining were a 1:300 dilution of MA6 and an overnight incubation.

Because MA6 tended to give non specific staining, it was found that after 20 min treatment with trypsin prior to immunostaining, staining was more distinct and background staining was reduced.

4.3. AUTOPSY TISSUE

4.3.1. HISTOLOGY

Microscopic examination of haematoxylin and eosin stained sections of A86-76 showed the epithelia which were separated from the lamina propria by the basal lamina. Throughout the lamina propria, mixed mucous and serous glands could be seen, occasionally with ducts leading to the epithelial surface. Lymphoid tissue was present throughout the anterior 2/3 of the specimen. No lymphoid follicles were identified. The tubal elevation was cartilaginous and covered by a thin layer of columnar or intermediate epithelium.

The anterior part of the nasopharynx was covered by pseudostratified ciliated columnar epithelium which extended over the area corresponding with the roof. Around the area of the openings of the eustachian tubes, the tubal elevations and the fossae of Rosenmüller, the columnar epithelium merged with intermediate epithelium which formed a wavy strip across the entire specimen. Towards the posterior third, that is the oral end of the nasopharynx, the intermediate epithelium gave way to stratified squamous epithelium. The divisions between the intermediate and the columnar and squamous epithelia were not distinct. There were always patches of each type of epithelium in the areas of the divisions. Figure 5 shows the distribution of epithelium in A86-76.

Specimens A87-368, A87-371 and A87-376 were taken to look closely at the histology and immunohistochemistry of the epithelia around the fossae of Rosenmüller, the tubal elevations and the eustachian openings, because it is in this region that the largest proportion of carcinomas are reported to arise. Although the exact distribution of epithelia varied from case to case, the anterior part was always covered by pseudostratified columnar epithelium and the posterior with stratified squamous epithelium. The area around the eustachian openings, the fossae of Rosenmüller and the tubal elevations in all four cases was pseudostratified ciliated columnar or intermediate epithelium or a mixture of both. See figures 6, 7 and 8 for diagrams of the distribution of epithelia in these autopsy specimens.

The three types of epithelium could be distinguished as follows:

Stratified squamous: six or seven or more layers thick. The basal cells, adjacent to the basal lamina, were cuboidal. The more mature cells nearer the epithelial surface were flattened, their nuclei

became small and pyknotic and the relative amount of cytoplasm increased. See figure 10.

Pseudostratified ciliated columnar: elongated cells with many mucin producing goblet cells - some areas had more goblet cells than others. The cell borders were always ciliated. See figures 11 and 12.

Intermediate: stratified into six or seven layers, the cells were cuboidal with virtually no flattening towards the surface and the nuclear size remained constant. Occasional remnants of goblet cells were seen. See figures 13 and 14.

4.3.2. HISTOCHEMISTRY

Mucin stains: Southgate's mucicarmine stained the acid mucins in the submucosal mucous glands and in the goblet cells of the pseudostratified ciliated columnar epithelium as illustrated in figure 12. Occasional goblet cells seen in the intermediate epithelium were also stained. Alcian blue stained more heavily at pH 2.5 than at pH 1.0, indicating an excess of acid over sulphated mucins in both the submucosal mucous glands and the epithelial goblet cells.

The phloxine tartrazine method is a useful general tissue stain. In the submucosa the collagen stained yellow and muscle red. Occasional patches of keratin in the squamous epithelium were phloxinophilic.

Gordon and Sweets' reticulin method clearly outlined the basal lamina which always forms the division between the epithelium and the submucosa. Reticulin was demonstrated as fine black fibres and collagen as coarse orange-brown fibres.

4.3.3. IMMUNOHISTOCHEMISTRY

MA6 staining of epithelium gave variable results. All three epithelial types would sometimes stain moderately and at other times would be negative. Mucous and serous glands were usually positive, as were myelin and striated muscle. Lymphocytes stained consistently strongly. Figure 15 shows MA6 staining of a section of columnar epithelium. Only the lymphocytes in the lamina propria are distinctly stained.

A86-76 was received after several weeks fixation in buffered formalin. Such prolonged fixation made it difficult to demonstrate cytokeratin consistently even after diastase digestion. The three later autopsy specimens of nasopharynx, A87-368, A87-371, and A87-376 were fixed for only 24 hours thus avoiding the 'over fixation' seen in A86-76.

AE1/AE3 stained the pseudostratified columnar epithelium strongly and the intermediate epithelium moderately. The basal cells and deeper third of the squamous epithelium stained, while the middle and upper thirds were negative. This is illustrated in figures 16 and 17. The simple cuboidal epithelium of the serous glands stained moderately strongly and of the mucous glands, weakly.

Immunostaining of stratified squamous and intermediate epithelium were both negative with anti cytokeratin 18. See figure 18. Columnar epithelium stained strongly with anti cytokeratin 18, but only in the upper parts of the cells. The intermediate and lower parts stained weakly or were negative. See figure 19.

Table 8 summarises the results of immunostaining the normal autopsy materials with the three monoclonal

antibodies.

4.4. INTRAEPITHELIAL LESIONS

4.4.1. IMMUNOHISTOCHEMISTRY

The various types of intraepithelial change seen in the 46 biopsies were often confined to small patches of epithelium which were surrounded by relatively normal epithelial tissue.

Immunostaining with AE1/AE3 antibodies gave the most interesting results. The general trend in these biopsies was that those areas showing intraepithelial changes stained less heavily than their adjacent more normal epithelia. Because of the heterogeneity of each biopsy, internal controls of normal epithelium could usually be found within each section so that comparisons of staining intensities could be made.

Patches of squamous metaplasia were always stained less strongly than the surrounding normal epithelium, whether it was columnar or intermediate. See figure 20. Squamous metaplasia may be difficult to identify in haematoxylin-eosin stained sections, as illustrated in figure 21, whereas with AE1/AE3 staining those areas can be seen fairly easily as in figure 22.

All hyperplastic epithelia stained less intensely than their normal counterparts.

Cells showing koilocytic change could be identified quite easily in haematoxylin-eosin sections (see figure 23) by their vacuolated cytoplasm and perinuclear halo. With AE1/AE3 the cytoplasm of these cells was unstained. See figure 24.

Those areas showing intraepithelial neoplasia were again less strongly stained with AE1/AE3 than the surrounding more normal epithelium although it was not possible to distinguish with any accuracy between nasopharyngeal intraepithelial neoplasia grades I, II and III by this method. See figure 25.

These results are summarised in table 9.

4.5. CARCINOMAS

4.5.1. HISTOCHEMISTRY

None of the carcinomas showed evidence of mucin production. The squamous cell carcinoma was encapsulated by reticulin fibres, as were 14 out of 27 undifferentiated carcinomas. See figure 26.

Phloxine tartrazine technique stained keratin red where it was present in large quantities in the case of keratinising squamous cell carcinoma. (See figures 27 and 28). Otherwise, phloxine tartrazine proved to be a good trichrome technique which would demonstrate tumour cells grey-blue clearly against the dark blue lymphocytes and yellow connective tissue (see figure 29), often more clearly than haematoxylin-eosin stained sections (figure 30).

4.5.2. IMMUNOHISTOCHEMISTRY

MA6 antibody gave variable results. Some tumours stained weakly and some showed moderately strong staining. However in all preparations there was some non specific background staining, even after treatment with trypsin prior to immunostaining. The antibody cross reacted with other tissue components besides tumour

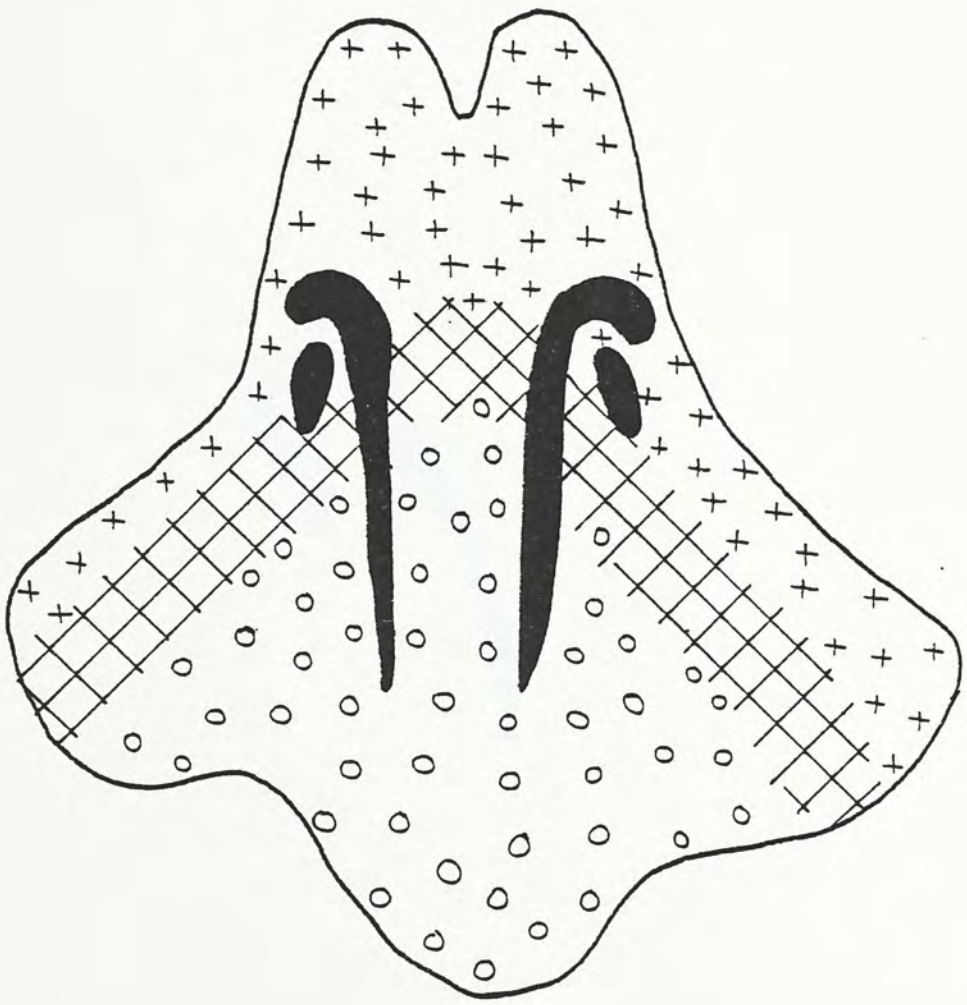
cells, such as myelin, striated muscle, lymphocytes and plasma cells which often made it difficult to identify the tumour cells. See figure 31.

AE1/AE3 stained the squamous cell carcinoma quite distinctly as in figure 32. Of the undifferentiated carcinomas, 26 out of 27 were positive with AE1/AE3. Examples are shown in figures 33 and 34. In biopsies where normal epithelia were available as internal controls, the tumour cells stained less densely than the normal tissues.

All of the carcinomas were negative with anti-cytokeratin 18.

A summary of the results of immunohistochemistry of the carcinomas is given in Table 10.

Figure 5. DISTRIBUTION OF NASOPHARYNGEAL EPITHELIA A36-76



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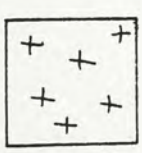
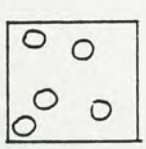


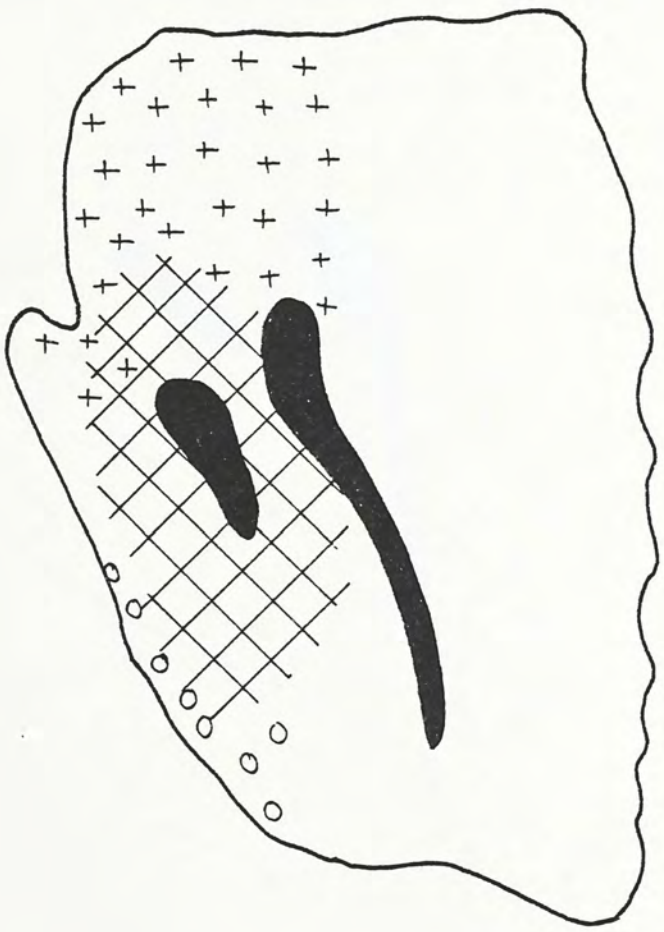
Pseudostratified ciliated columnar epithelium

Intermediate epithelium



Stratified squamous epithelium

Figure 6. DISTRIBUTION OF NASOPHARYNGEAL EPITHELIA A87-368



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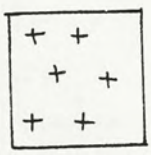
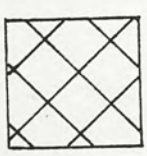
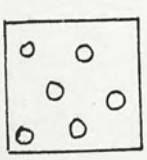


Pseudostratified ciliated columnar epithelium

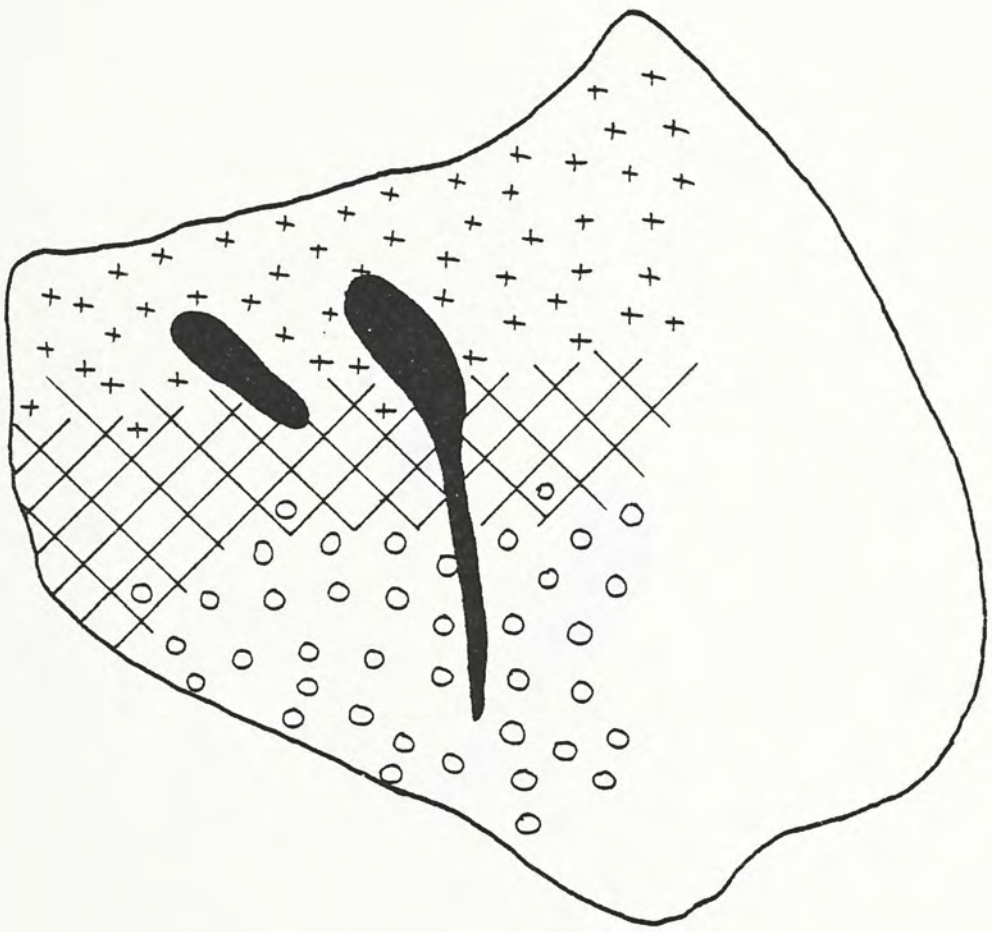


Intermediate epithelium



Stratified squamous epithelium

Figure 7. DISTRIBUTION OF NASOPHARYNGEAL EPITHELIA A87-371



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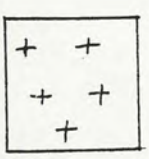
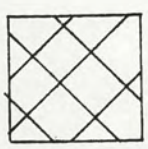
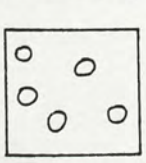


Pseudostratified ciliated columnar epithelium

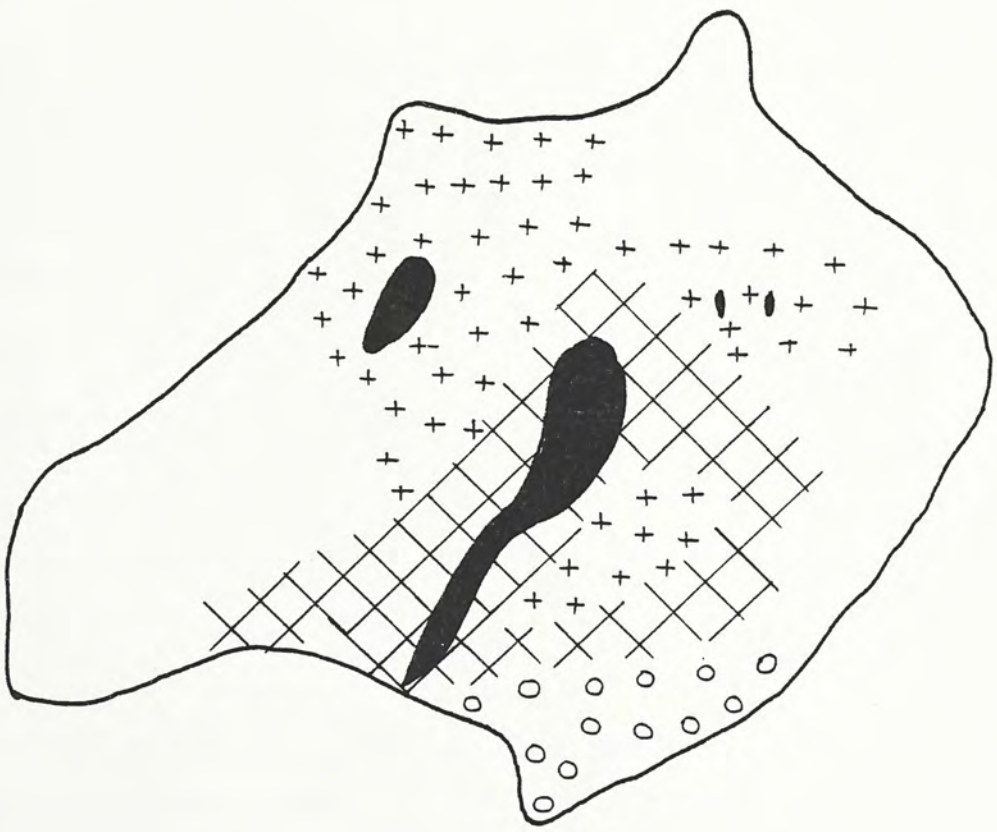


Intermediate epithelium



Stratified squamous epithelium

Figure 8. DISTRIBUTION OF NASOPHARYNGEAL EPITHELIA A37-376



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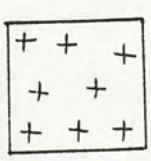
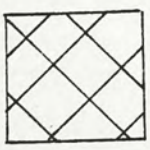
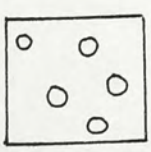


Pseudostratified ciliated columnar epithelium



Intermediate epithelium



Stratified squamous epithelium

TABLE 5. CHEQUERBOARD TITRATION FOR OPTIMUM DILUTIONS OF BIOTINYLATED RABBIT ANTI MOUSE AND STREPTAVIDIN-BIOTIN-PEROXIDASE.

B-RAM DILUTION	1:200	1:250	1:300	1:400
Stra-B-Px DILUTION				
1:100	+++*	++	++	++
1:200	+++*	+++	++	++
1:300	+++*	+++	++	+
1:400	+++	+++	++	+
1:500	++	++	+	+

NOTES:

1. B-RAM = biotinylated rabbit anti mouse Igs
2. Stra-B-Px = streptavidin-biotin-peroxidase
3. Control tissue: buffered formalin fixed paraffin section of oesophageal carcinoma
4. Trypsin incubation time: 1 hour
5. Primary antibody: AE1/AE3 1:150 for 30 min
6. Intensity of staining: + = weak, ++ = medium, +++ = strong
7. * denotes background staining.

TABLE 6. AE1/AE3 OPTIMUM DILUTION AND INCUBATION TIME

TIME	30 MIN	1 HR	2 HR	OVERNIGHT
DILUTION				
1:100	+	+	++	++
1:200	+	+	++	+++
1:300	+	+	++	+++
1:400	+	+	++	++

NOTES:

1. Control tissue: buffered formalin fixed paraffin section of oesophageal carcinoma
2. Trypsin incubation time: 1 hour
3. Biotinylated rabbit anti mouse: 1:250 for 30 min
4. Streptavidin-biotin-peroxidase: 1:200 for 30 min
5. Intensity of staining: + = weak, ++ = medium, +++ = strong.

TABLE 5. CHEQUERBOARD TITRATION FOR OPTIMUM DILUTIONS OF BIOTINYLATED RABBIT ANTI MOUSE AND STREPTAVIDIN-BIOTIN-PEROXIDASE.

B-RAM DILUTION	1:200	1:250	1:300	1:400
Stra-B-Px DILUTION				
1:100	+++*	++	++	++
1:200	+++*	+++	++	++
1:300	+++*	+++	++	+
1:400	+++	+++	++	+
1:500	++	++	+	+

NOTES:

1. B-RAM = biotinylated rabbit anti mouse Igs
2. Stra-B-Px = streptavidin-biotin-peroxidase
3. Control tissue: buffered formalin fixed paraffin section of oesophageal carcinoma
4. Trypsin incubation time: 1 hour
5. Primary antibody: AE1/AE3 1:150 for 30 min
6. Intensity of staining: + = weak, ++ = medium, +++ = strong
7. * denotes background staining.

TABLE 6. AE1/AE3 OPTIMUM DILUTION AND INCUBATION TIME

TIME	30 MIN	1 HR	2 HR	OVERNIGHT
DILUTION				
1:100	+	+	++	++
1:200	+	+	++	+++
1:300	+	+	++	+++
1:400	+	+	++	++

NOTES:

1. Control tissue: buffered formalin fixed paraffin section of oesophageal carcinoma
2. Trypsin incubation time: 1 hour
3. Biotinylated rabbit anti mouse: 1:250 for 30 min
4. Streptavidin-biotin-peroxidase: 1:200 for 30 min
5. Intensity of staining: + = weak, ++ = medium, +++ = strong.

TABLE 7. ANTI-CYTOKERATIN 18 OPTIMUM DILUTION AND INCUBATION TIME

TIME	2 HOURS	OVERNIGHT
DILUTION		
1:5	+	+++
1:10	+	+++
1:15	+	+++
1:20	+	++

NOTES:

1. Control tissue: buffered formalin fixed paraffin section of renal biopsy
2. Trypsin incubation time: 20 min
3. Biotinylated rabbit anti mouse: 1:250 for 30 min
4. Streptavidin-biotin-peroxidase: 1:200 for 30 min
5. Intensity of staining: + = weak, ++ = medium, +++ = strong.

TABLE 8. IMMUNOHISTOCHEMISTRY OF NORMAL NASOPHARYNX

TISSUE / ANTIBODY	SQUAMOUS EPITHELIUM	INTERMEDIATE EPITHELIUM	COLUMNAR EPITHELIUM
AE1/AE3	+*	++	+++
CK 18	-	-	+++**
MA6	-/+	-/+	-/+

TISSUE / ANTIBODY	MUCOUS GLANDS	SEROUS GLANDS
AE1/AE3	+	++
CK 18	+	+++
MA6	++	++

* = Lower third stains only. Middle and upper thirds negative.

** = Upper parts of cells stain only. Middle and lower parts weak or negative.

NOTES:

1. MA6 also stained other tissues including lymphocytes, plasma cells, myelin and striated muscle.
2. Intensity of staining: - = negative, -/+ = variable, + = weak, ++ = medium, +++ = strong.

TABLE 9. IMMUNOHISTOCHEMISTRY OF INTRAEPITHELIAL LESIONS

ANTIBODY	CYTOKERATIN 18	AE1/AE3*
LESION		
HYPERPLASIA	++**	++
METAPLASIA	-	++
KOILOCYTES	-	-
NPIN	-	++

* = AE1/AE3 always stained less intensely than in nomal epithelia.

** = Cytokeratin 18 was positive only with columnar epithelium.

NOTES:

1. NPIN = nasopharyngeal intraepithelial neoplasia.

2. Intensity of staining: - = negative, ++ = medium.

TABLE 10. IMMUNOHISTOCHEMISTRY OF CARCINOMAS

ANTIBODY	MA6	CK 18	AE1/AE3*
LESION			
SCC (N = 1)	+	-	++
UC (N = 27)	-/+	-	++ (26/27)

* = AE1/AE3 always stained less intensely than associated normal epithelia.

CK 18 = anti cytokeratin 18.

SCC = squamous cell carcinoma.

UC = undifferentiated nasopharyngeal carcinoma.

5. DISCUSSION

Preliminary work with the modification of the anticomplement (ACIF) test for EBNA was reasonably successful. Smears of Raji cells fixed in acetone-methanol and buffered formalin showed brown granular staining in the nuclei which was comparable with the fluorescence pattern seen in the ACIF test. Non specific cytoplasmic staining was minimal when the primary antiserum was diluted to 1:200 or 1:400. Periodate-lysine-paraformaldehyde (PLP) fixed smears were negative.

Sections of the pelleted Raji cells fixed briefly (1 hour) and routinely processed to paraffin wax also showed positive granular staining in cell nuclei of those fixed in acetone-methanol and buffered formalin. This granularity was taken to be EBNA. Morphological preservation was much better in the acetone-methanol fixed cells than those fixed in buffered formalin. Again the PLP-fixed cells were negative. PLP is a fixative that has been used for immunoelectron microscopy and for immunohistochemistry of nervous tissue.[220,221] It was therefore surprising that the cells were completely negative for EBNA after fixation with it.

The positive results with the sections of acetone-methanol or buffered formalin fixed and paraffin processed Raji cells indicated that what was assumed to be EBNA in the smears had not been destroyed by longer fixation and the paraffin processing schedule.

The xenograft, however, failed to give any positive staining. Time was spent modifying the procedure by altering dilutions and incubation times, but results were consistently negative.

The nuclei of the tumour cells were negative in the frozen sections from an NPC case but some lymphocytes stained strongly. This was attributed to the second layer immunoreagent, a rabbit anti human IgG, which reacted with anti EBNA antibodies in the primary antiserum. Naturally the anti human IgG reacted with any IgG-containing plasma cells and lymphocytes. It was decided that the ACIF modification in this form would not be easily applicable to paraffin processed biopsy material as there is no reliable way of blocking IgG-containing plasma cells and because tumour cells in the xenograft and the frozen section had not stained. The antiserum may have lacked the high titre and avidity necessary for successful immunohistochemistry on paraffin sections. It is possible that the extended fixation time of six hours plus the paraffin processing may have altered the antibody binding site of EBNA.

The next stage of the investigation was a retrospective histochemical and immunohistochemical study of the epithelium of the normal nasopharynx and of nasopharyngeal biopsies which showed intraepithelial changes and malignancies.

Histology of four autopsy specimens of nasopharynx confirmed Ali's early study of the histology of the nasopharyngeal epithelium.[9] The anterior part (the nasal end) is covered with stratified squamous epithelium while the posterior part (the oral end) has a covering of pseudostratified columnar epithelium. At the junction of these two epithelial types is a distinct zone of intermediate epithelium. This epithelium is stratified into 6 or 8 layers. All the cells are cuboidal, they do not mature into the flattened cells seen in the stratified squamous epithelium. Intermediate epithelium covers the area around the Eustachian openings and the fossae of

Rosenmüller and the upper part of the salpingopharyngeal folds. These areas have been noted in many studies to be common sites of origin of NPC.[2,10,13]

Results of Southgate's stain indicated that all the mucins in both the epithelium and the lamina propria are acid mucins. By staining with alcian blue at varying pH it is possible to roughly distinguish between sulphated (which stain at pH 1.0) and acid (which stain at pH 2.5) mucins. Alcian blue staining in this study stained more heavily at pH 2.5 than at pH 1.0, suggesting an excess of acid over sulphated mucins in the epithelium and the lamina propria. This confirmed Tock and Tan's[118] more detailed study of the mucins of the nasopharynx. They found more sialidase resistant sialomucins (sialomucins are among the group of acid mucins) than sulphated mucins.

None of the tumours were mucin producing, so demonstration of mucins would not generally be a useful aid to diagnosis of malignancies.

Phloxine tartrazine stain demonstrated occasional patches of keratin in squamous epithelium where it was present in large enough quantities to be stained. Phloxine tartrazine was found to be a useful trichrome technique, demonstrating tumour cells grey-blue against the dark blue lymphocytes and yellow stroma.

Silver impregnation for reticulin fibres clearly showed the basal laminae. Some of the tumour biopsies showed islets of tumour cells that were encapsulated by reticulin fibres. Liang[97] suggested that those tumours showing encapsulation tended to be well differentiated and did not invade and metastasise, while those without encapsulation were poorly differentiated and more

frequently invaded the cranial base and metastasised.

The immunogen for MA6 antibody was an immunoprecipitate obtained by counterimmunoelectrophoresis of serum from an NPC patient against an extract of lysed Raji cells. The purpose of using this immunogen may have been to try and produce an antibody against one of the EBV antigens. MA6 has been identified to react with a 55Kd glycoprotein called B-lymphocyte carcinoma cross-reacting antigen (BLCa). BLCa has turned out to be an antigen which is widely distributed in tissues. It occurs on B-lymphocytes as well as on carcinomas including NPC.[174] This study showed that MA6 also reacted with several other sites including squamous epithelium, myelin, muscle and blood vessels.

MA6 did not stain the NPC cells with sufficient intensity or selectivity to be useful as a tumour marker. B-lymphocytes were most heavily demonstrated. NPCs are often associated with a heavy lymphocyte infiltrate, which, if stained with MA6, could obscure small foci of tumour cells.

It has already been shown that immunohistochemical identification of cytokeratin is so far the most reliable means of distinguishing NPC from other tumours of the area of the nasopharynx. The next part of the study involved the immunolocalisation of cytokeratin in the tissues under study.

AE1/AE3 is a 'broad spectrum' anti cytokeratin. Its suppliers, Hybritech, claim it will demonstrate all types of epithelium. Distinct and reproducible patterns of reactivity with both normal and abnormal nasopharyngeal tissues emerged. Pseudostratified columnar epithelium stained more intensely than intermediate epithelium which

in turn stained more heavily than stratified squamous epithelium. Only the basal cells of stratified squamous epithelium tended to stain, while the middle and upper layers were unstained.

Hyperplastic epithelium of all types stained positively with AE1/AE3. Areas of squamous metaplasia stained less intensely than the surrounding more normal epithelium, which was often intermediate epithelium. Small patches of squamous metaplasia can be difficult to identify in haematoxylin-eosin stained sections, so staining with AE1/AE3 could be a useful aid for its identification. Certain carcinomas are believed to arise from within areas of squamous metaplasia, for example squamous carcinoma of the bronchus.[101] Whether or not squamous metaplasia in the nasopharynx is a precursor to carcinoma is not known.

The squamous and intermediate epithelium of the nasopharynx occasionally shows cells with a similar appearance to the koilocytes which have been described in other sites and have been attributed to viral infections.[102,103,104,105,106] These cells have a prominent nucleolus, a perinuclear halo and vacuolated cytoplasm, but a viral association in the nasopharynx has not been confirmed. In this study, koilocyte cytoplasm was clearly unstained with AE1/AE3. The cells were easily identifiable, particularly in intermediate epithelium, where the surrounding unaffected cells were quite darkly stained with AE1/AE3.

Areas of intraepithelial neoplasia stained less intensely than adjacent normal epithelium. It was difficult to identify a distinct pattern of reactivity with nasopharyngeal intraepithelial neoplasia, but in general, the degree of staining decreased with the increasing

severity of the neoplastic change.

Twenty-seven out of twenty-eight carcinomas stained positively with AE1/AE3. Staining intensities varied, but using adjacent normal epithelia where available, as internal controls, the tumour cells tended to stain less intensely than the normal epithelia. The one specimen of squamous cell carcinoma showed variable staining intensities within the tumour itself. Well differentiated areas of tumour were less intensely stained than comparatively undifferentiated areas. A parallel may be drawn between these appearances with AE1/AE3 and those of normal squamous epithelium, where the well differentiated upper layers of squamous cells stain less intensely than the relatively undifferentiated deeper layers.

Cytokeratin 18 is one of the low molecular weight (45Kd) cytokeratin proteins which occurs only in simple epithelia. In this investigation, anti-cytokeratin 18 reacted only with the upper parts of pseudostratified columnar epithelial cells. This is in concurrence with an investigation of the cytokeratins of the epithelium of the upper respiratory tract by Nagle et al.[236] using a murine monoclonal antibody, RGE-53, which reacts with cytokeratin 18. Immunofluorescence studies of the nasopharynx showed the same pattern of reactivity with the pseudostratified columnar epithelium as in this study. The cuboidal epithelium of mucous and serous glands in the lamina propria was also positive. These are all simple epithelia. The more complex intermediate and stratified squamous epithelia did not stain with anti-cytokeratin 18. Areas of hyperplastic columnar epithelium were demonstrated, but all other intraepithelial lesions were unstained. Cytokeratin 18 was negative with all carcinomas.

This study has been an attempt to define the epithelial cell populations of the nasopharynx by histochemical means. The conventional histochemical methods confirmed the results of several earlier studies, on the type and distribution of mucins and the distribution of reticulin fibres. Such histochemistry has not been able to solve the problems of histological classification and histogenesis of the tumours.

Immunohistochemistry has shown that it may be possible to define the nasopharyngeal epithelia by identifying suitable antigen markers within the tissue.

MA6 antibody, which identifies the widely distributed BLCa antigen, is probably not specific enough a marker for epithelia or for nasopharyngeal carcinoma cells to be of use in such a study.

AE1/AE3 is a useful broad spectrum immunoreagent for the recognition of epithelial cells and epithelial derived tumours. The cytoplasm of koilocytes was unstained. It stained all but one of the NPCs in the study. It identified areas of squamous metaplasia, which may in future prove to be of diagnostic significance in NPC if the role of squamous metaplasia in the nasopharynx is established and it also stained intraepithelial neoplasia. Overall, AE1/AE3 stained all normal epithelia more heavily than those that were undergoing pathological changes, both benign and malignant, which indicates that the total expression of cytokeratins is reduced in abnormal epithelia.

CK 18 only stained the tips of the columnar cells. It was negative with all of the tumours. It is known that some carcinomas express cytokeratin subsets that are very similar to their normal precursor cells, for example, adenocarcinoma of colon. Others, such as breast cancers

produce a subset which is different from the corresponding normal tissues.[165,167] It is not known whether those cytokeratins produced by NPC cells are the same or different to those produced by the precursor epithelial cells.

If NPC were known to express the same cytokeratin subset as its cells of origin, then the negative results with anti-cytokeratin 18 would indicate that the tumours do not arise from the columnar epithelium. Conversely, if the cytokeratin subset is different in the normal epithelial cells than in the tumour cells, then these results do not help to answer the question of the histogenesis of NPC.

Further study of cytokeratin expression by the nasopharyngeal epithelium may help to establish the precursor cell type of NPC. If it were possible to establish the cytokeratin subsets expressed by each of the epithelial types and by the carcinomas, then comparisons of these subsets may give an indication of the cell or cells of origin of the tumours. However, as has been suggested by Batsakis et al,[10] the intermediate epithelium provides the original cell, the composition of the cytokeratins of intermediate cells could well show a combination of the subsets seen in the stratified squamous and the columnar epithelium. This has been shown to be the case with other 'unusual' epithelia, such as the transitional epithelium of the bladder and tracheal pseudostratified columnar epithelium.[162,166] Intermediate epithelium would have no well defined cytokeratin characteristics for comparison with those of the carcinomas. At present, the functional significance of the complex distribution of cytokeratins is unclear.[236]

ACKNOWLEDGMENTS

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Finally, to Dr Peter Lewis and Mr Michael Shum who allowed me time to work on the project, I should like to extend my thanks.

APPENDIX 1

MISCELLANEOUS FIXATIVES, SOLUTIONS AND BUFFERS

1. PERIODATE-LYSINE-PARAFORMALDEHYDE FIXATIVE[220,221]

Stock reagents:

A. Lysine Phosphate Buffer

To 0.2M lysine-HCl add 0.1M dibasic sodium phosphate until pH = 7.4. Dilute with 0.1M sodium phosphate buffer, pH 7.4 to make 0.1M lysine.

B. Paraformaldehyde

Dissolve, with heat, 8g paraformaldehyde in 100ml water. Add a few drops of 10% sodium hydroxide.

C. Sodium meta-periodate (mol wt 213.9)

Working Fixative Solution:

3 parts A
1 part B
2.139g C

Make up to 100ml with distilled water. pH drops from 7.4 to 6.2. 3mm tissue blocks can be fixed without pH adjustment at 4°C for 3 hours.

2. BUFFERED FORMALIN

10ml formalin (40% HCHO)
0.4g sodium phosphate, monobasic
0.65g sodium phosphate, dibasic
Make up to 100ml with distilled water.

3. ACETONE-METHANOL

Prepare fresh each time.
Equal volumes of fresh acetone and methanol.

4. CHROME ALUM-GELATINE COATED SLIDES[222]

1. Dissolve 3.0g gelatine in 1 litre distilled water, heated to approx 60°C
2. Add 0.5g chromium potassium sulphate (chrome alum). The solution will turn blue
3. Add several thymol crystals
4. Filter a small portion while hot into a 100ml beaker, keeping the stock hot
5. Place several slides, one at a time, into the beaker
6. When slides are dry, collect and store at room temperature in a slide box.

5. PHOSPHATE BUFFERED SALINE, pH 7.1-7.4

Stock Solution:

435g NaCl
13.6g KH_2PO_4 (anhydrous)
5 litres distilled water

For Use:

Make a 1:10 dilution in distilled water. Adjust pH to between 7.1 and 7.4.

6. 0.05M TRIS BUFFERED SALINE, pH 7.6

Stock Solution:

81g NaCl
6.0g Tris (hydroxymethyl) methylamine
1N HCl
1 litre distilled water

For Use: Make a 1:10 dilution in distilled water.

7. 0.05M TRIS-HCl BUFFER pH 7.6

(for DAB-H_2O_2)

Stock Solutions:

A. 0.2M Tris:

24.2g Tris (hydroxymethyl) methylamine

1 litre distilled water

B. 0.2M HCl:

17ml conc. HCl

1 litre distilled water

For Use:

250ml A + 192ml B. Make up to 1 litre with distilled water.

8. DIAMINOBENZIDINE-H_2O_2 (DAB-H_2O_2)

Peroxidase substrate. Prepare fresh each time.

50mg diaminobenzidine tetrahydrochloride (Sigma[212])

100ml 0.05M Tris-HCl buffer, pH 7.6

Just before use, add 0.05ml fresh 1% H_2O_2.

9. TRYPSIN[210,211]

This solution should be prepared immediately before use and not re-used. The solution and the slides should be warmed to 37°C before use.

0.1g trypsin (pancreatic type II, Sigma[212])

100ml 0.1% $CaCl_2$

Adjust pH to 7.8 with NaOH.

10. MAYER'S HAEMATOXYLIN

1.0g haematoxylin

50g potassium alum

0.2g sodium iodate

1.0g citric acid

50g chloral hydrate

1 litre distilled water

Dissolve in order. Boil for 5 mins. Cool and filter.

APPENDIX 2

HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

1. MAYER'S HAEMATOXYLIN AND EOSIN

Method:

1. Take sections to water
2. Stain in Mayer's haematoxylin, 5 min
3. Wash and blue in running tapwater, 5 min
4. Counterstain in 1% aqueous eosin, 3 min
5. Rinse quickly in tapwater
6. Dehydrate in alcohol, clear in xylene and mount in synthetic resin.

Results:

Nuclei - blue; cytoplasm, red blood cells, muscle, collagen - shades of pink and red.

2. SOUTHGATE'S MUCICARMINE[225]

Preparation of Stain:

Grind 1g carmine and place in a large conical flask. Add 100ml 50% alcohol and mix. Add 1g aluminium hydroxide, mix, and add 0.5g anhydrous aluminium chloride. Mix and boil for 3 min. Cool, filter and store at 4°C.

Method:

1. Take sections to water
2. Stain in Mayer's haematoxylin for 5 min
3. Wash and blue in running tapwater
4. Stain with mucicarmine for 20 min
5. Wash in distilled water
6. Dehydrate in alcohol, clear in xylene and mount in synthetic resin.

Results:

Nuclei - blue; mucins - red.

3. ALCIAN BLUE pH 2.5 and pH 1.0[229]

Staining Solutions:

pH 2.5:

1g alcian blue 8GX
3ml acetic acid
100ml distilled water

pH 1.0:

1g alcian blue 8GX
100ml 0.1N HCl

Method:

1. Take sections to water
2. Stain with appropriate filtered alcian blue solution, 10 min
3. Wash in running water, 5 min
4. Stain nuclei in 0.25% acid fuchsin in 1% acetic acid, 30 sec
5. Rinse quickly in distilled water
6. Dehydrate in alcohol, clear in xylene and mount in synthetic resin.

Results:

At pH 2.5: nuclei - red; weakly acid sulphated mucins - blue; sulphated mucins stain weakly or not at all.

At pH 1.0: nuclei - red; sulphated mucins - blue; acid mucins stain weakly or not at all.

5. PHLOXINE-TARTRAZINE[230]

Staining Solutions:

Phloxine:

0.5g phloxine

0.5g calcium chloride

100ml distilled water

Tartrazine:

Saturated solution of tartrazine in 2-ethoxyethanol

Method:

1. Take sections to water
2. Stain in Mayer's haematoxylin, 5 min
3. Wash and blue in running tapwater
4. Stain in phloxine, 20 min
5. Rinse in water, then in 2-ethoxyethanol
6. Stain in tartrazine, checking with microscope until only red blood cells are strongly stained with phloxine
7. Wash in 2-ethoxyethanol
8. Clear in xylene and mount in synthetic resin.

Results:

Nuclei - blue; red blood cells - bright red; muscle and keratin - red; other tissues - yellow.

6. GORDON AND SWEETS' SILVER IMPREGNATION FOR RETICULIN[231]

Solutions:

Acidified potassium permanganate:

47.5ml 0.25% potassium permanganate

2.5ml 3% sulphuric acid

Ammoniacal silver:

To 5ml 10% silver nitrate add conc ammonia drop by drop with frequent mixing until the formed precipitate just redissolves. Add 5ml of 3.1% sodium hydroxide and mix. A precipitate will form which gradually dissolves upon the addition of ammonia, drop by drop as before. Stop when there only a few precipitate granules remaining. Make up the final volume to 50ml with distilled water.

Method

1. Take sections to water
2. Treat with acidified permanganate, 5 min
3. Wash off and bleach with 5% oxalic acid
4. Rinse in distilled water
5. Treat with 3% ferric ammonium sulphate, 5 min
6. Wash well in distilled water
7. Treat with ammoniacal silver, 4-5 sec
8. Wash well in distilled water
9. Reduce in 10% formalin in tapwater, 30 sec
10. Wash and treat with 5% sodium thiosulphate, 3 min
11. Dehydrate in alcohol, clear in xylene and mount in synthetic resin.

Results:

Reticulin - black; collagen - yellow-brown; background - unstained.

APPENDIX 3

IMMUNOHISTOCHEMICAL METHODS

1. MODIFIED METHOD FOR EBNA

1. Take sections to water and rinse in phosphate buffered saline (PBS)(see appendix 1) or bring smears to room temp and rinse in PBS
2. Block endogenous peroxidase activity (if necessary) with fresh 3% H_2O_2 in methanol
3. Wash in PBS and incubate with primary antiserum diluted to 1:100 at 37°C for 60 min
4. Wash in 3 changes of PBS over 5 min
5. Incubate with biotinylated goat anti human IgG diluted 1:200 at room temp for 30 min
6. Wash in 3 changes of PBS over 5 min
7. Incubate with streptavidin-biotin-peroxidase, diluted 1:200 for 45 min
8. Wash in 3 changes of PBS over 5 min
9. Develop peroxidase in DAB-H_2O_2 for 5-10 min or until optimum staining is achieved (see appendix 1)
10. Wash in tapwater
11. Stain nuclei in Mayer's haematoxylin, 30 sec
12. Wash and blue in running tapwater
13. Dehydrate in alcohol, clear in xylene and mount in synthetic resin.

A positive result is shown by brown staining.

All dilutions are prepared in PBS with 1% normal goat serum.

Negative controls:

1. Replace primary antiserum with serum which is negative for EBV antibodies (step 3)
2. Omit secondary antibody (step 5)

2. IMMUNOSTAINING WITH AE1/AE3[232]

1. Take sections to absolute alcohol
2. Block endogenous peroxidase activity in 3% H_2O_2 in methanol
3. Wash in 0.05M Tris buffered saline pH 7.6 (TBS)(see appendix 2)
4. Treat with trypsin at 37°C for 1 hour (see appendix 1)
5. Wash in 3 changes of TBS over 5 min
6. Incubate in AE1/AE3 diluted 1:300 overnight at room temp
7. Wash in 3 changes of TBS over 5 min
8. Incubate in biotinylated rabbit anti mouse Igs diluted 1:250 for 30 min
9. Wash in 3 changes of TBS over 5 min
10. Incubate in streptavidin-biotin-peroxidase, diluted 1:200 for 30 min
11. Wash in 3 changes of TBS over 5 min
12. Develop peroxidase in DAB-H_2O_2 (see appendix 1), for 5 - 10 min, checking microscopically until optimum staining is achieved
13. Wash in tapwater
14. Stain nuclei in Mayer's haematoxylin, 1 min
15. Wash and blue in running tapwater
16. Dehydrate in alcohol, clear in xylene and mount in synthetic resin.

A positive result is shown by brown staining.

All dilutions are prepared in TBS and all incubations are carried out at room temperature.

Negative control:
Replace primary antibody with non-immune mouse serum (step 6).

3. IMMUNOSTAINING WITH ANTI-CYTOKERATIN 18[233]

1. Take sections to absolute alcohol
2. Block endogenous peroxidase activity in 3% H_2O_2 in methanol
3. Wash in 0.05M Tris buffered saline pH 7.6 (TBS)(see appendix 1)
4. Treat with trypsin (see appendix 1) at 37°C for 20 min
5. Wash in 3 changes of TBS over 5 min
6. Incubate in anti CK 18, diluted 1:15 overnight
7. Wash in 3 changes of TBS over 5 min
8. Incubate in biotinylated rabbit anti mouse Igs diluted 1:250 for 30 min
9. Wash in 3 changes of TBS over 5 min
10. Incubate in streptavidin-biotin-peroxidase, diluted 1:200 for 30 min
11. Wash in 3 changes of TBS over 5 min
12. Develop peroxidase in DAB-H_2O_2 for 5 - 10 min (see appendix 1) or until optimum staining is achieved
13. Wash in tapwater
14. Stain nuclei in Mayer's haematoxylin for 1 min
15. Wash and blue in running tapwater
16. Dehydrate in alcohol, clear in xylene and mount in synthetic resin

A positive result is shown by brown staining.

Anti CK 18 is diluted in PBS, pH 7.2 with 1% bovine serum albumin. All other dilutions are prepared in TBS. All incubations are at room temperature.

Negative control:
Replace primary antibody with non immune mouse serum (step 6).

4. IMMUNOSTAINING WITH MA6 ANTIBODY[174]

1. Take sections to absolute alcohol
2. Block endogenous peroxidase activity in 3% H_2O_2 in methanol
3. Wash in 0.05M Tris buffered saline (TBS)(see appendix 2)
4. Treat with trypsin (see appendix 1) at 37°C for 20 min
5. Wash in 3 changes of TBS over 3 min
6. Incubate in MA6 diluted 1:150 for 30 min
7. Wash in 3 changes of TBS over 5 min
8. Incubate in biotinylated anti mouse Igs diluted 1:250 for 30 min
9. Wash in 3 changes of TBS over 5 min
10. Incubate in streptavidin-biotin-peroxidase diluted 1:200 for 30 min
11. Wash in 3 changes of TBS over 5 min
12. Develop peroxidase in DAB-H_2O_2 (see appendix 1) for 5-10 min or until optimum staining is achieved
13. Wash in tapwater
14. Stain nuclei in Mayer's haematoxylin for 1 min
15. Wash and blue in running tapwater
16. Dehydrate in alcohol, clear in xylene and mount in synthetic resin.

A positive result is shown by brown staining.

All dilutions are prepared in TBS and all incubations are at room temperature.

Negative control:
Replace primary antiserum with non immune mouse serum (step 6).

REFERENCES

1. Nasopharyngeal Carcinoma: Etiology and Control.(1978) Proceedings of an International Symposium held in Kyoto, Japan, 4-6 April 1977. Ed. de-Thé, G. & Ito, Y. IARC Scientific Publication no. 20. International Agency for Research on Cancer, Lyon, France.
2. Prasad, U. (1972) Cancer of the nasopharynx. Journal of the Royal College of Surgeons of Edinburgh, 17, 108-117.
3. Grant, J.C.B.(1980) Grant's Method of Anatomy: by Regions, Descriptive and Deductive. 10th edn. Williams and Wilkins, Baltimore.
4. Gray, H. (1980) Gray's Anatomy, 36th edn. Longman, Edinburgh.
5. Textbook of Human Anatomy. (1976) 2nd edn. Ed. Hamilton, W.J. MacMillan, London.
6. The John's Hopkins Atlas of Anatomy. (1977) Text ed: Zuidema, G.O. John's Hopkins University Press, Baltimore.
7. Leeson, T.S. & Leeson, C.R. (1981) Histology. 4th edn. W.B. Saunders Co, Philadelphia.
8. Friedman, I. (1986) The Nasopharynx. In: Systemic Pathology, volume 1. Nose, Throat and Ears. p. 131-160. Ed: Friedman, I. Churchill Livingstone, Edinburgh.
9. Ali, M.Y., (1965) Histology of the human nasopharyngeal mucosa. Journal of Anatomy, 99, 657-672.
10. Batsakis, J.G., Solomon, A.R. & Rice, D.H. (1981) The pathology of the head and neck: carcinoma of the nasopharynx, part 11. Head and Neck Surgery, 3, 511-524.
11. Kanagasuntheram, R. & Ramsbotham, M. (1968) Development of the human nasopharyngeal epithelium. Acta

Anatomica, 70, 1-13.

12. Bryant, W.S. (1916) The transition of the ciliated epithelium of the nose into the squamous epithelium of the pharynx. Journal of Anatomy. 50, 172-176.
13. Yeh, S. (1962) A histological classification of carcinomas of the nasopharynx with a critical review as to the existence of lymphoepitheliomas. Cancer, 15, 895-920.
14. Lin, H.S., Lin, C.S., Yeh, S. & Tu, S.M. (1969) Fine Structure of nasopharyngeal carcinoma with special reference to the anaplastic type. Cancer, 23, 390-405.
15. Pearse, A.G.E. (1985) Histochemistry: Theoretical and Applied, Volume 2, 4th edn. Churchill Livingstone, Edinburgh.
16. Clifford, P. (1970) On the epidemiology of nasopharyngeal carcinoma. International Journal of Cancer, 5, 287-309.
17. Ho, J.H.C. (1978) An epidemiologic and clinical study of nasopharyngeal carcinoma. International Journal of Radiation Oncology, Biology and Physics, 4, 183-198.
18. de-Thé, G. (1981) The Chinese epidemiological approach of nasopharyngeal carcinoma research and control. Yale Journal of Biology and Medicine, 54, 33-39.
19. Cancer Incidence in Hong Kong. (1980) Cancer Registry, Queen Elizabeth Hospital, Kowloon, Hong Kong.
20. Li, C.C., Yu, M.C. & Henderson, B.E. (1985) Some epidemiologic observations of nasopharyngeal carcinoma in Guangdong, People's Republic of China. National Cancer Institute Monograph, 69, 49-52.
21. de Thé, G. (1979) Demographic studies implicating the virus in the causation of Burkitt's lymphoma: prospects for nasopharyngeal carcinoma. In: The

Epstein-Barr Virus, p. 417-437. Ed. Epstein, M.A. & Achong, B.G. Springer-Verlag, Berlin.

22. Muir, C.S. (1972) Epidemiology and Etiology. Journal of the American Medical Association, 220, 393-394.
23. Nielsen, N.H., Mikkelsen, F. & Hansen, J.P.H. (1977) Nasopharyngeal cancer in Greenland. Acta Pathologica, Microbiologica, et Immunologica Scandinavica. Section A, Pathology, 85, 850-858.
24. Blot, W.J., Lanier, A., Fraumeni J.F. & Bender T.R. (1975) Cancer mortality among Alaskan Natives 1960-69. Journal of the National Cancer Institute, 55, 547-554.
25. Ho, H.C. (1976) Epidemiology of nasopharyngeal carcinoma. GANN Monograph on Cancer Research, 18, 49-61.
26. Chan, S.H., Day, N.E., Kunaratnam, N. Chia, K.B. & Simons, M.J. (1983) HLA and nasopharyngeal carcinoma in Chinese - a further study. International Journal of Cancer, 32, 171-176.
27. Wildy, P. (1985) Herpes viruses: a background. British Medical Bulletin, 41, 339-334.
28. Epstein, M. A. & Achong, B.G. (1979) Discovery and general biology of the virus. In: The Epstein-Barr Virus, p. 1-22. Ed. Epstein, M.A. & Achong, B.G. Springer-Verlag, Berlin.
29. Epstein, M.A. & Barr, Y. M. (1964) Cultivation in vitro of human lymphoblasts from Burkitt's malignant lymphoma. Lancet, I, 252-253.
30. Epstein, M.A., Achong, B. G. & Barr, Y.M. (1964) Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet, I, 702-703.
31. Henle, G. & Henle, W. (1966) Immunofluorescence in cells derived from Burkitt's lymphoma. Journal of Bacteriology, 91, 1248-1256.
32. Epstein, M.A. & Achong, B.G. (1968) Observations on the nature of the herpes type Epstein-Barr virus in

cultured Burkitt lymphoblasts, using a specific immunofluorescence test. Journal of the National Cancer Institute, 40, 609-621.

33. Henle, G. & Henle, W. (1968) Relation of Burkitt's tumor associated herpes-type virus to infectious mononucleosis. Proceedings of the National Academy of Science of the USA, 59, 94-101.

34. Burkitt, D. (1958) A sarcoma involving the jaws in African children. British Journal of Surgery, 46, 218-223.

35. Ten Seldam, R.E.J., Cooke, R. & Atkinson, L. (1966) Childhood lymphoma in the territories of Papua and New Guinea. Cancer, 19, 437-446.

36. Wright, D.H. (1963) Cytology and histochemistry of the Burkitt lymphoma. British Journal of Cancer, 17, 50-55.

37. Wright, D. A lymphoma syndrome in tropical Africa. International Review of Experimental Pathology, 2, 97-102.

38. Miller, G. (1982) Burkitt lymphoma. In: Viral infections of humans: epidemiology and control, 2nd edn. p 599-620. Ed. Evans A.S. Plenum, New York.

39. Zur Hausen, H., Schulte-Holthausen, H., Klein, G., Henle, W., Henle, G., Clifford, P. & Santesson, L. (1970) EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature, 228, 1056-1058.

40. Lindahl, T., Klein, G., Reedman, B.M., Johansson, B. & Singh, S. (1974) Relationship between Epstein-Barr virus (EBV) and the EBV-determined nuclear antigen (EBNA) in Burkitt lymphoma biopsies and other lymphoproliferative malignancies. International Journal of Cancer, 13, 764-772.

41. Henle, G., Henle, W., Clifford, P., Diehl, V., Kafuko, G.W., Kirya, B.G., Klein, G., Morrow, R.H., Manube, G., Pike, P., Tukei, P.M. & Zeigler, J.L.

(1969) Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. Journal of the National Cancer Institute, 43, 1147-1157.

42. Zeigler, J.L., Andersson M., Klein, G. & Henle, G. (1976) Detection of Epstein-Barr virus DNA in American Burkitt's lymphoma. International Journal of Cancer, 17,701-706.

43. Neiderman, J.C., McCollum, R.W., Henle, G. & Henle, W. (1968) Infectious mononucleosis. Journal of the American Medical Association, 203, 205-209.

44. Niederman, J.C., Evans, A.S., Subramanyan, M.S. & McCollum, R.W. (1970) Prevalence, incidence and persistence of EB virus antibody in young adults. New England Journal of Medicine, 282, 361-365.

45. Henle, W., Henle, G. & Lennette, E.T. (1979) The Epstein-Barr virus. Scientific American, 241, 40-51.

46. Pereira, M.S., Field, A.M., Blake, J.M., Rodgers, F.G., Bailey, L.A. & Davies, J.R. (1972) Evidence for oral excretion of EB virus in infectious mononucleosis. Lancet, I, 710-711.

47. Old, L.J., Boyse, E.A., Oettgen, H.F., de Harven, E., Geering, G., Williamson, B. & Clifford, P. (1966) Precipitating antibody in human serum to an antigen present in cultured Burkitt's lymphoma cells. Proceedings of the National Academy of Science of the USA. 56, 1699-1704.

48. Henle, W. & Henle, G. (1969) The relation between the Epstein-Barr virus and infectious mononucleosis, Burkitt's lymphoma and carcinoma of the post-nasal space. East African Medical Journal, 46, 402-406.

49. Henle, W., Henle, G., Ho H.C., Clifford, P., de Schryver, A., de-Thé, G., Diehl, V. & Klein, G. (1970) Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma, other head and neck neoplasms and control groups. Journal of the National Cancer Institute, 44, 225-231.

50. de Schryver, A., Friberg, S., Klein, G., Henle, G., Henle, W., de-Thé, G., Clifford, P. & Ho, H.C. (1969) Clinical and Experimental Immunology, 5, 443-459.

51. de-Thé, G., Ho, J.H.C. & Muir, C.S. (1982) Nasopharyngeal Carcinoma. In: Viral Infections of Humans, 2nd edn, p 621-652. Ed. Evans, A.S. Plenum, New York.

52. Anonymous. (1979) Some progress with nasopharyngeal carcinoma. Lancet, I, 959-960.

53. Prasad, U. & Gogusev, J. (1978) Intracisternal tubular inclusions in nasopharyngeal carcinoma. Journal of Laryngology and Otolaryngology, 92, 979-989.

54. Nadol, J.B. Viral particles in nasopharyngeal carcinoma. Laryngoscope, 87, 1932-1937.

55. Wolf, H., Zur Hausen, H. & Becker, V. (1973) EB viral genomes in epithelial nasopharyngeal carcinoma cells. Nature New Biology, 244, 245-247.

56. Desgranges, C., Wolf, H., de-Thé, G., Shanmugaratnam, K., Cammoun, N., Ellouz, R., Klein, G., Lennert, K., Munoz, N. & Zur Hausen, H. Nasopharyngeal carcinoma. X. Presence of Epstein-Barr genomes in separated epithelial cells of tumours in patients from Singapore, Tunisia and Kenya. International Journal of Cancer, 16, 7-15.

57. Andersson-Anvret, M., Forsby, N., Klein, G. & Henle, W. (1977) Relationship between the Epstein-Barr virus and undifferentiated nasopharyngeal carcinoma: correlated nucleic acid hybridisation and histopathological examination. International Journal of Cancer, 20, 486-494.

58. Raab-Traub, N., Flynn, K., Pearson, G., Huang, A., Lanier, A. & Pagano, J. (1987) The differentiated form of NPC contains EBV DNA. International Journal of Cancer, 39, 25-29.

59. Huang, D.P., Ho, J.H.C., Henle, W. & Henle, G. (1974) Demonstration of Epstein-Barr virus associated antigen in nasopharyngeal carcinoma cells from fresh biopsies. International Journal of Cancer, 14, 580-588.

60. Huang, D.P., Ho, H.C., Henle, W., Henle, G., Saw, D. & Lui, M. (1978) Presence of EBNA in nasopharyngeal carcinoma and control patient tissues related to EBV serology. International Journal of Cancer, 22, 266-274.

61. Miller, G., Grogan, E., Fischer, D.K., Niederman, J.C., Schooley, R.T., Henle, W., Lenoir, G. & Liu, C-R. (1985) Antibody responses to EBV nuclear antigens defined by gene transfer. New England Journal of Medicine, 312, 750-755.

62. Wara, W.M., Wara, D.W., Phillips, T.L. & Ammann, A.J. (1975) Elevated IgA in carcinoma of the nasopharynx. Cancer, 35, 1313-1315.

63. Ho, H.C., Ng, M.H., Kwan, H.C. & Chau, J.C.W. (1976) Epstein-Barr virus specific IgA and IgG serum antibodies in nasopharyngeal carcinoma. British Journal of Cancer, 34, 655-660.

64. Henle, G. & Henle, W. (1976) Epstein-Barr virus specific IgA serum antibodies as an outstanding feature of NPC. International Journal of Cancer, 17, 1-7.

65. Ho, H.C., Kwan, H.C., Ng, M.H. & de-Thé, G. (1978) Serum IgA antibodies to Epstein-Barr virus capsid antigen preceding symptoms of NPC. Lancet, I, 436.

66. Feighny, R.J., Berch, E.H. & Pagano, J.S. (1981) Epstein-Barr virus polypeptides: identification of early proteins and their synthesis and glycosylation. Journal of Virology, 39, 651-655.

67. Grogan, E.A., Summers, W.P., Dowling, S., Shedd, D., Gradoville, L. & Miller, G. (1983) Two Epstein-Barr viral nuclear neoantigens distinguished by gene

transfer, serology and chromosome binding. Proceedings of the National Academy of Science of the USA, 80, 7650-7653.

68. Luka, J., Jörnvall, H. & Klein, G. (1980) Purification and biochemical characterisation of the Epstein-Barr virus determined nuclear antigen and an associated protein with a 53,000 dalton subunit. Journal of Virology, 35, 592-602.

69. Spelsberg, T.C., Sculley, T.B., Pickler, G.M., Gilbert, J.A. & Pearson, G.R. (1982) Evidence for two classes of chromatin associated Epstein-Barr virus determined nuclear antigen. Journal of Virology, 43, 555-565.

70. Hennessy, K. & Kieff, E. One of two Epstein-Barr virus nuclear antigens contains a glycine-alanine copolymer domain. Proceedings of the National Academy of Science of the USA, 80, 5665-5669.

71. Shanmugaratnam, K. & Sobin, H. (1978) Histological typing of upper respiratory tract tumours. International Histological Typing of Tumours, no. 19, p. 32-33. WHO, Geneva.

72. Neel, H.B., Lanier, A.P., Taylor, W.F., Huang, A.T., Goepfert, H.H., Hyams, V.J., Pilch, B.Z., Levine, P.H., Henle, G. & Henle, W. (1980) Anti-EBV serologic tests for nasopharyngeal carcinoma. Laryngoscope, 90, 1981-1990.

73. Wolf, H., Zur Hausen, H., Klein, G., Becker, V., Henle, G. & Henle, W. (1975) Attempts to detect virus-specific DNA sequences in human tumors. III. Epstein-Barr viral DNA in non-lymphoid nasopharyngeal carcinoma cells. Medical Microbiology and Immunology, 161, 15-21.

74. Andersson-Anvret, M., Forsby, N., Klein, G., Henle, W. & Biörklund A. (1979) Relationship between the Epstein-Barr virus genome and nasopharyngeal carcinoma in Caucasian patients. International Journal

of Cancer, 23, 762-767.

75. Pearson, G.R., Wieland, L.H., Neel, H.B., Taylor, W., Earle, J., Mulroney, S.E., Goepfert, H., Lanier, A., Talvot, M.L., Pilch, B., Goodman, M., Huang, A., Levine, P.H., Hyams, V., Moran, E., Henle, G. & Henle, W. (1983) Application of EBV serology to the diagnosis of North American nasopharyngeal carcinoma. Cancer, 51, 260-268.

76. Zeng, Y., Zhang, L.G., Li, H.Y., Jan, M.G., Zhang, Q., Wu, Y.C., Wang, Y.S. & Su, G.R. (1982) Serological mass survey for detection of nasopharyngeal carcinoma in Wuzhou city, China. International Journal of Cancer, 29, 139-141.

77. Zeng, Y., Zhang, L.G., Wu, Y.C., Huang, Y.S., Li, J.Y., Wang, Y.B., Jiang, M.K., Fang, Z. & Meng, N.N. (1985) Prospective studies on nasopharyngeal carcinoma in Epstein-Barr virus IgA/VCA antibody positive persons in Wuzhou city, China. International Journal of Cancer, 36, 545-547.

78. Jondal, M. & Klein, G. (1973) Surface markers on human B and T lymphocytes. Journal of Experimental Medicine, 138, 1365-1378.

79. Fingeroth, J.D., Weiss, J.J., Tedder, T.F., Strominger, J.L., Biro, P.A. & Fearon, D.T. (1984) Epstein-Barr virus receptor of human B-lymphocytes is the C3d receptor CR2. Proceedings of the National Academy of Science of the USA, 81, 4510-4514.

80. Young, L.S., Clark, D., Sixbey, J.W. & Rickinson, A.B. (1986) Epstein-Barr virus receptors on human pharyngeal epithelia. Lancet, I, 240-242.

81. Lemon, S.M., Hutt, L.M., Shaw, J.E., Li, J.L.H. & Pagano, J.S. (1977) Replication of EBV in epithelial cells during infectious mononucleosis. Nature, 268, 268-270.

82. Sixbey, J.W., Nedrud J.G., Raab-Traub, N., Hanes, R.A. & Pagano, J.S. (1984) Epstein-Barr virus

replication in oropharyngeal epithelial cells. New England Journal of Medicine, 310, 1225-1230.

83. Wolf, H., Haus, M. & Wilmes, E. (1984) Persistence of Epstein-Barr virus in the parotid gland. Journal of Virology, 51, 795-798.

84. Lung, M.L., Lam, W.K., So, S.Y., Lam, W.P. Chan, K.H. & Ng, M.H. (1985) Evidence that the respiratory tract is a major reservoir for Epstein-Barr virus. Lancet, I, 889-892.

85. Leyvraz, S., Henle, W., Chahinian, A.P., Perlmann, C., Klein, G., Gordon, R.E., Rosenblum, M. & Holland, J.F. (1985) Association of Epstein-Barr virus with thymic carcinoma. New England Journal of Medicine, 312, 1296-1299.

86. Saemundsen, A.K., Albeck, H., Hansen, J.P.H., Nielsen, N.H., Anvret, M., Henle, W., Henle, G., Thomsen, K.A., Kristensen, H.K. & Klein, G. (1981) Epstein-Barr virus in nasopharyngeal and salivary gland carcinomas of Greenland Eskimos. British Journal of Cancer, 46, 721-728.

87. Lin, T.M., Yang, C.S., Tu, S.M., Chen, C.J., Kuo, K.C. & Hirayama, T. (1979) Interaction of factors associated with cancer of the nasopharynx. Cancer, 44, 1419-1423.

88. Hirayama, T. & Ito, Y. (1981) A new view of the etiology of nasopharyngeal carcinoma. Preventive Medicine, 10, 614-622.

89. Zeng, Y., Zhong, J.M., Mo, Y.K. & Miao, X.C. (1983) Epstein-Barr virus early antigen induction in Raji cells by Chinese medicinal herbs. Intervirology, 19, 201-204.

90. Armstrong, R.W., (1983) Salted fish and inhalents as risk factors for nasopharyngeal carcinoma in Malaysian Chinese. Cancer Research, 43, 2967-2970.

91. Yu, M.C., Ho, J.H.C., Ross, R.K. & Henderson, B.E. (1981) Nasopharyngeal carcinoma in Chinese - salted

fish or inhaled smoke? Preventive Medicine, 10, 15-24.

92. Yu, M.C., Ho, J.H.C., Henderson, B.E. & Armstrong, R.W. (1985) Epidemiology of nasopharyngeal carcinoma in Malaysia and Hong Kong. National Cancer Institute Monograph, 69, 203-207.

93. Yu, M.C., Ho, J.H.C., Lai, S.H. & Henderson, B.E. (1986) Cantonese style salted fish as a cause of nasopharyngeal carcinoma: report of a case-control study in Hong Kong. Cancer Research, 46, 956-961.

94. Huang, D.P., Ho, J.H.C. & Gough, T.A. (1978) Analysis for volatile nitrosamines in salt preserved foodstuffs traditionally consumed by Southern Chinese. In: Nasopharyngeal Carcinoma: Etiology and Control. Ed. de-Thé, G. & Ito, Y. IARC Scientific Publication no. 20, p 309-314. International Agency for Research on Cancer, Lyon, France.

95. Huang, D.P., Ho, J.H.C., Saw, D. & Teoh, T.B. (1978) Carcinoma of the nasal and paranasal regions in rats fed Cantonese salted marine fish. In: Nasopharyngeal carcinoma: Etiology and Control. Ed: de-Thé, G. & Ito, Y. IARC Scientific Publication no. 20, p 315-328. International Agency for Research on Cancer, Lyon, France.

96. Shanmugaratnam, K. (1978) Histological typing of nasopharyngeal carcinoma. In: Nasopharyngeal Carsinoma: Etiology and Control. Ed: de-Thé, G & Ito, Y. IARC Scientific Publication no. 20. International Agency for Research on Cancer, Lyon, France.

97. Liang. P-C., Ch'en, C-C., Chu, C-C., Hu, Y-F., Chu, H-M. & Tsung, Y-S. (1962) The histopathologic classification, biologic characteristics and histogenesis of nasopharyngeal carcinoma. Chinese Medical Journal, 81, 629-658.

98. Teoh, T.B. (1957) Epidermoid carcinoma of the nasopharynx among Chinese: a study of 31 necropsies.

Journal of Pathology and Bacteriology, 73, 451-465.
99. Prasad, U. (1974) Cells of origin of nasopharyngeal carcinoma: an electron microscopic study. Journal of Laryngology and Otology, 88, 1087-1094.
100. Prehn, R.T. (1980) In: Principles of Pathobiology, p200-211. Ed. Hill, R.B. & La Via, M.F. Oxford University Press.
101. Slack. J.M.W. (1986) Epithelial metaplasia and the second anatomy. Lancet, II, 268-272.
102. Jenson, A.B., Sommers, S., Payling-Wright, C., Pass, F., Link, C.C. & Lancaster, W.D. (1982) Human papillomavirus. Frequency and distribution in plantar and common warts. Laboratory Investigation, 47, 491-497.
103. Greenspan, D., Greenspan, J.S., Conant, M., Petersen, V., Silverman, S. & de Souza, Y. (1984) Oral 'hairy' leucoplakia in male homosexuals: evidence of association with both papillomavirus and a herpes-group virus. Lancet, II, 831-834.
104. Crum, C.P., Ikenberg, H., Richart, R.M. & Gissman, L. (1984) Human papillomavirus type 16 and early cervical neaplasia. New England Journal of Medicine, 310, 880-883.
105. Greenspan, J.S., Greenspan, D., Lennette, E.T., Abrams, D.I., Conant, M.A., Petersen, V. & Freese, U.K. (1985) Replication of Epstein-Barr virus within the epithelial cells of oral 'hairy' leucoplakia, an AIDS-associated lesion. New England Journal of Medicine, 313, 1564-1571.
106. Löning, T., Henke, R-P., Reichart, P. & Becker, J. (1987) In situ hybridisation to detect Epstein-Barr virus DNA in oral tissues of HIV-infected patients. Virchows Archiv. A, Pathological Anatomy and Histopathology, 412, 127-133.
107. Koss, L.G. (1979) Diagnostic Cytology, 3rd edn. Lippincott.
108. Lee, J.C.K. & Suen, M.W.M. (1986) Intraepithelial

neoplasia in mucosa of human nasopharyngeal carcinoma. Proceedings of the XVIth International Congress of the Academy of Pathology, Vienna, Austria.

109. Shanmugaratnam, K., Chan, S.H., de-Thé, G., Goh, J.E.H., Khor, T.H., Simons, M.J. & Tye, C.Y. (1979) Histopathology of nasopharyngeal carcinoma. Correlations with epidemiology, survival rates and other biological characteristics. Cancer, 44, 1029-1044.
110. Svoboda, D.J. Pathologic classification and fine structure. (1972) Journal of the American Medical Association, 220, 394-408.
111. Micheau, C., Rilke, F. & Pilotti, S. (1978) Proposal for a new histopathological classification of the carcinomas of the nasopharynx. Tumori, 64, 513-518.
112. Wieland, L.H. (1978) The histopathological spectrum of nasopharyngeal carcinoma. In: nasopharyngeal carcinoma: Etiology and Control, p. 41-50. Ed. de-Thé, G. & Ito, Y. IARC Scientific Publications no. 20. International Agency for Research on Cancer, Lyon, France.
113. Sugano, H., Sakamoto, G., Sawaki, S. & Hirayama, T. (1978) Histopathological types of nasopharyngeal carcinoma in a low risk area: Japan. In: Nasopharyngeal Carcinoma: Etiology and Control, p. 27-39. Ed. de-Thé, G. & Ito, Y. IARC Scientific Publications no 20. International Agency for Research on Cancer, Lyon, France.
114. Cammoun, M., Ellouz, R., Behi, J. & Ben Attia, R. (1978) Histological types of nasopharyngeal carcinoma in an intermediate risk area. In: nasopharyngeal Carcinoma: Etiology and Control, p. 13-26. Ed. de-Thé, G. & Ito, Y. IARC Scientific Publications no. 20. International Agency for Research on Cancer, Lyon, France.
115. Krueger, G.R.F., Kottarides, S.D., Wolf, H.,

Ablashi, D.V., Sesterhenn, K. & Bertram, G. (1981) Histological types of nasopharyngeal carcinoma as compared to EBV serology. Anticancer Research, 1, 187-194.

116. Hsu, H.C., Chen, C.L., Hsu, M.M., Lynn, T.C., Tu, S.M. & Huang, S.C. (1987) Pathology of nasopharyngeal carcinoma. Proposal of a new classification correlated with prognosis. Cancer, 59, 945-951.

117. Micheau, C. (1986) What's new in histological classification and recognition of nasopharyngeal carcinoma. Pathology Research and Practice, 181, 249-253.

118. Tock, E.P.C. & Tan, N.T.S. (1969) A histochemical study of the mucins of the adult human nasopharynx. Journal of Anatomy, 104, 81-92.

119. Lennert, K., Kaiserling, E. & Mazzanti, T. (1978) Diagnosis and differential diagnosis of lymphoepithelial carcinoma in lymph nodes: histological, cytological and electron-microscopic findings. In: Nasopharyngeal Carcinoma: Etiology and Control, p. 51-64. Ed. de-Thé, G. & Ito, Y. IARC Scientific Publications no. 20. International Agency for Research on Cancer, Lyon, France.

120. Micheau, C. (1978) L'apport de la cyto-histo-enzymologie (CHE) au diagnistic des tumeurs. Archives d'Anatomie et de Cytologie Pathologiques, 26, 190-196.

121. Prathap, K., Looi, L.M. & Prasad, U. (1984) Localised amyloidosis in nasopharyngeal carcinoma. Histopathology, 8, 27-34.

122. Reedman, B.M. & Klein, G. (1973) Cellular localisation of an Epstein-Barr virus (EBV) complement fixing antigen in producer and non-producer lymphoblastoid cell lines. International Journal of Cancer, 11, 499-520.

123. Zeng, Y., Pi, G. & Zhao, W. (1980) Detection of EB

virus nuclear antigen (EBNA) by anti complement immunoenzymatic method. Acta Academiae Medicinae Sinicae, 2, 162-168.

124. Schlegel, R., Banks-Schlegel, S., McLeod, S. & Pinkus, G.S. (1980) Immunoperoxidase localisation of keratin in human neoplasms. American Journal of Pathology, 101, 41-50.

125. Madri, J.A. & Barwick, K.W. (1982) An immunohistochemical study of nasopharyngeal neoplasms using keratin antibodies. American Journal of Surgical Pathology, 6, 143-149.

126. Zeigels-Weissman, J., Nadji, M., Penneys, N.S. & Morales, A.R. (1984) Prekeratin immunohistochemistry in the diagnosis of undifferentiated carcinoma of the nasopharyngeal type. Archives of Pathology Laboratory Medicine, 108, 588-589.

127. Gusterson,B.A., Mitchell, D.P., Warburton,M.J. & Carter, R.L. (1983) Epithelial markers in the diagnosis of nasopharyngeal carcinoma: an immunocytochemical study. Journal of Clinical Pathology, 36, 628-631.

128. Shi, S-R., Goodman, M., Bahn, A.K., Pilch, B.Z., Chen, L.B. & Sun, T-T. (1984) Immunohistochemical study of nasopharyngeal carcinoma using monoclonal keratin antibodies. American Journal of Pathology, 117, 53-63.

129. Taxy, J.B., Hidvegi, D.F. & Battifora, H. (1985) Nasopharyngeal carcinoma: antikeratin immunohistochemistry and electron microscopy. American Journal of Clinical Pathology, 83, 320-325.

130. Bosq, J., Gatter, K.C., Micheau, C. & Mason, D.Y. (1985) Role of immunohistochemistry in diagnosis of nasopharyngeal tumours. Journal of Clinical Pathology, 38, 845-848.

131. Nakai, M. & Mori, M. (1986) Immunohistochemical distribution of monoclonal antibodies against keratin

in papillomas and carcinomas from oral and nasopharyngeal regions. Oral Surgery, Oral Medicine, Oral Pathology, 62, 292-302.

132. Oppedal, B.R., Böhler, P.J., Marton, P.F. & Brandtzaeg, P. (1987) Carcinoma of the nasopharynx. Histopathological examination with supplementary immunohistochemistry. Histopathology, 11, 1161-1169.

133. Heyderman,E. (1983) Tumour Markers. In: Immunocytochemistry, p 274-293. Ed. Polak, J.M. & Van Noorden, S. John Wright & Sons Ltd, Bristol, England.

134. Pinkus, G.S. & Kurtin, P.J. (1985) Epithelial membrane antigen - a diagnostic discriminant in surgical pathology. Human Pathology, 16, 929-940.

135. Heyderman E., Steele, K. & Ormerod, M.G. (1979) A new antigen on the epithelial membrane: its immunoperoxidase localisation in normal and neoplastic tissue. Journal of Clinical Pathology, 32, 35-39.

136. Sloane, J.P. & Ormerod, M.G. (1981) Distribution of epithelial membrane antigen in normal and neoplastic tissues and its value in diagnostic tumour pathology. Cancer, 47, 1786-1795.

137. Sloane, J.P., Hughes, F. & Ormerod, M.G. (1983) An assessment of the value of epithelial membrane antigen and other epithelial markers in solving diagnostic problems in tumour histopathology. Histochemical Journal, 15, 645-654.

138. Pinkus, G.S., Etheridge, C.L. & O'Connor, E.M. (1986) Are keratin proteins a better tumour marker than epithelial membrane antigen? American Journal of Clinical Pathology, 85, 269-277.

139. Thomas, P. & Battifora, H. (1987) Keratins versus epithelial membrane antigen in tumour diagnosis. Human Pathology, 18, 728-734.

140. Gold, P. & Freedman, S.O. (1965) Demonstration of tumor specific antigens in human colonic carcinomata

by immunological tolerance and absorption techniques. Journal of Experimental Medicine, 121, 439-462.

141. Gold, P. & Freedman, S.O. (1965) Specific carcinoembryonic antigens of the human digestive system. Journal of Experimental Medicine, 122, 467-481.
142. Huitric, E., Laumonier. R., Burtin, P., Von Kleist S. & Chavanel, G. (1976) An optical and ultrastructural study of the localisation of carcinoembryonic antigen (CEA) in normal and cancerous human rectocolonic mucosa. Laboratory Investigation, 34, 97-107.
143. Primus, F.J., Sharkey, R.M., Hansen, H.J. & Goldenberg, D.M. (1978) Immunoperoxidase detection of carcinoembryonic antigen. Cancer, 42, 1540-1545.
144. Hamada, Y., Yamamura, M., Hioki, K., Yamamoto, M., Nagura, H. & Watanabe, K. (1985) Immunohistochemical study of carcinoembryonic antigen in patients with colorectal cancer. Cancer, 55, 136-141.
145. Nap, M., Keuning, H., Burtin, P., Oosterhuis, J.W. & Fleuren, G. (1984) CEA and NCA in benign and malignant breast tumours. American Journal of Clinical Pathology, 82, 526-534.
146. Talerman, A., Linderman, J., Keivit-Tyson, P.A. & Dröge-Droppert, C. (1979) Demonstration of calcitonin and carcinoembryonic antigen (CEA) in medullary carcinoma of the thyroid (MCT) by immunoperoxidase technique. Histopathology, 3. 503-501.
147. Sharkey, R.M., Primus, F.J. & Goldenberg, D.M. (1980) Comparison of the sensitivity of the indirect, antibody conjugated and the triple bridge immunoperoxidase methods for immunohistochemical detection of carcinoembryonic antigen. Histochemistry, 66, 35-42.
148. Bychkov, V., Rothman, M. & Bardawil, W.A. (1983) Im-

munocytochemical localisation of carcinoembryonic antigen, alpha fetoprotein and human chorionic gonadotrophin in cervical neoplasia. American Journal of Clinical Pathology, 79, 414-420.

149. Pinkus, G.S. (1982) Diagnostic immunocytochemistry of paraffin embedded tissues. Human Pathology, 13, 411-415.

150. Kahn, H.J., Marks, A., Thom, H. & Bauma, R. (1983) Role of antibody to S-100 protein in diagnostic pathology. American Journal of Clinical Pathology, 79, 341-347.

151. Polak, J.M. & Bloom, S.R. (1983) Regulatory peptides. In: Immunocytochemistry, p. 184-211. Ed. Polak, J.M. & Van Noorden, S. John Wright & Sons Ltd, Bristol, England.

152. Lauriola, L., Michetti, F., Sentinelli, S. & Cocchia, D. (1984) Detection of S-100 labelled cells in nasopharyngeal carcinoma. Journal of Clinical Pathology, 37, 1235-1238.

153. Nomori, H., Watanabe, S., Nakajima, T., Shimosato Y. & Kameya, T. (1986) Histiocytes in nasopharyngeal carcinoma in relation to prognosis. Cancer, 57, 100-105.

154. Hammar, S., Bockus, D., Remington, F. & Bartha, M. (1986) The widespread distribution of Langerhans cells in pathologic tissues. Human Pathology, 17, 894-905.

155. Flavell, D.J., Jones, D.B. & Wright, D.H. (1987) Identification of tissue histiocytes on paraffin sections by a new monoclonal antibody. Journal of Histochemistry and Cytochemistry, 35, 1217-1226.

156. Wick, M.R., Swanson, P.E. & Manivel, J.C. (1987) Placental-like alkaline phosphatase reactivity in human tumours. Human Pathology, 18, 946-954.

157. Nagle, R.B., McDaniel, K.M., Clark, V.A. & Payne, C.M. (1983) The use of antikeratin antibodies in the

diagnosis of human neoplasms. American Journal of Clinical Pathology, 79, 458-466.

158. Klinge, E.M., Sylvestre, Y.R., Freedberg, I.M. & Blumberg, M. (1987) Evolution of keratin genes: different protein domains evolve by different pathways. Journal of Molecular Evolution, 24, 319-329.

159. Moll, R., Franke, W.W. & Schiller, D.L. (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell, 31, 11-24.

160. Lazarides, E. (1980) Intermediate filaments as mechanical integrators of cellular space. Nature, 283, 249-256.

161. Lazarides, E. (1982) Intermediate filaments: a chemically heterogeneous developmentally regulated class of proteins. Annual Review of Biochemistry, 51, 219-250.

162. Sun, T-T., Eichner, R., Schermer, A., Cooper, D., Nelson, W.G. & Weiss, R.A. (1984) Classification, expression and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. In: Cancer Cells, 1. The Transformed Phenotype, p. 169-176. Ed. Levine, A.J., Vande Woude, G.F., Topp, W.C. & Watson, J.D.

163. Tseng, S.C.G., Jarvinen, M.J., Nelson, W.G., Huang, J.W., Woodcock-Mitchell, J. & Sun, T-T. (1982) Correlation of specific keratins with different types of epithelial differentiation: monoclonal antibody studies. Cell, 30, 361-372.

164. Woodcock-Mitchell, J., Eichner, R., Nelson, W.G. & Sun, T-T. (1982) Immunolocalisation of keratin polypeptides in human epidermis using monoclonal antibodies. Journal of Cell Biology, 95, 580-588.

165. Fuchs, E., Grace, M.P., Kim, K.H. & Marchuk, D. (1984) Differential expression of two classes of keratins in normal and malignant epithelial cells

and their evolutionary conservation. In: Cancer Cells, 1. The Transformed Phenotype, p. 169-176. Ed. Levine, A.J., Vande Woude, G.F., Topp, W.C. & Watson, J.D.

166. Schlegel, R., Banks-Schlegel, S. & Pinkus, G.S. (1980) Immunohistochemical localisation of keratin in normal human tissues. Laboratory Investigation, 42, 91-96.

167. Moll, R., Krepler, R. & Franke, W.W. (1983) Complex cytokeratin polypeptide patterns observed in certain human carcinomas. Differentiation, 23, 256-269.

168. Osborn, M. & Weber, K. (1983) Biology of disease. Tumour diagnosis by intermediate filament typing: a novel tool for surgical pathology. Laoratory Investigation, 48, 372-394.

169. Gabbiani, G., Kapanci, Y., Barazzone, P. & Franke, W.W. (1981) Immunochemical identification of intermediate-sized filaments in human neoplastic cells. American Journal of Pathology, 104, 206-216.

170. Gown, A.M. & Vogel, A.M. (1985) Monoclonal antibodies to human intermediate filament proteins. American Journal of Clinical Pathology, 84, 413-424.

171. Knight, J., Gusterson, B., Jones, R.R., Landells, W. & Wilson, P. (1985) Monoclonal antibodies specific for subsets of epidermal keratins. Journal of Pathology, 145, 341-354.

172. Makin, C.A., Bobrow, L.G. & Bodmer, W.F. (1984) Monoclonal antibody to cytokeratin for use in routine histopathology. Journal of Clinical Pathology, 37, 975-983.

173. Lauder, I., Holland, D., Mason, D.Y., Gowland, G. & Cunliffe, W.J. (1984) Identification of large cell undifferentiated tumours in lymph nodes using leucocyte common and keratin antibodies. Histopathology, 8, 259-272.

174. Chan, K.H., Yip, T.C., Ng, W.L. & Ng, M.H. (1985) A

shared antigenic determinant between human B-lymphocytes and carcinomas (BLCa). International Journal of Cancer, 36, 329-336.

175. Yip, T.C., Chan, K.H. & Ng, M.H. (1987) Characterisation of a human B-lymphocyte cross reacting antigen (BLCa) in B-lymphocytes identified by two murine monoclonal antibodies. International Journal of Cancer, 39, 452-458.

176. Yip, T.C., Chan, K.H., Choy, D., Chan, C.W. & Ng, M.H. (1987) Characterisation of a murine monoclonal antibody defined B-lymphocyte carcinoma cross-reacting antigen (BLCa) from nasopharyngeal carcinoma tissues. International Journal of Cancer, 39, 449-451.

177. Chan, K.H., Yip, T.C., Choy, D., Chan, C.W., Zeng, Y. & Ng, M.H. (1987) Evaluation of monoclonal antibodies for the detection of exfoliative nasopharyngeal carcinoma cells. International Journal of Cancer, 39, 445-448.

178. Van Noorden, S. & Polak, J.M. (1983) Immunocytochemistry today. In: Immunocytochemistry, p 11-42. Ed. Polak, J.M. & Van Noorden, S. John Wright & Sons Ltd, Bristol, England.

179. Pearse, A.G.E. (1980) Histochemistry: Theoretical and Applied, Volume 1, 4th edn. Churchill livingstone, Edinburgh.

180. Coons, A.H., Creech, H.J. & Jones, R.N. (1941) Immunological properties of an antibody containing a fluorescent group. Proceedings of the Society of Experimental Biology and Medicine, 47, 200-202.

181. Coons, A.H. & Kaplan, M.H. (1950) Localisation of antigen in tissue cells. II. Improvements in a method for the detection of an antigen by means of a fluorescent antibody. Journal of Experimental Medicine, 91, 1-13.

182. Coons, A.H., Ledec, E.H. & Connolly, J.M. (1955)

Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. Journal of Experimental Medicine, 102, 49-60.

183. Riggs, J.L., Seiwald, R.J., Burckhalter, J.H., Downs, C.M. & Metcalf, T.G. (1958) Isothiocyanate compounds as fluorescent labelling agents for immune serum. American Journal of Pathology, 34, 1081-1097.

184. Nakane, P.K. & Pierce, G.B. (1966) Enzyme labelled antibodies: preparation and application for the localisation of antigens. Journal of Histochemistry and Cytochemistry, 14, 929-931.

185. Zeng, Y., Shen, S., Pi, G., Ma, J., Zhang, Q., Zhao, M. & Dong, H. (1981) Application of anticomplement immunoenzymatic method for the detection of EBNA in carcinoma cells and normal epithelial cells from the nasopharynx. In: Cancer Campaign, volume 5. Nasopharyngeal Carcinoma, p. 237-245. Ed. Grundmann et. al. Gustav Fischer Verlag, Stuttgart.

186. Pi, G., Zeng, Y., Zhao, W. & Zhang, Q. (1981) Development of an anticomplement immunoenzyme test for the detection of EB virus nuclear antigen (EBNA) and antibody to EBNA. Journal of Immunological Methods, 44, 73-78.

187. Heyderman, E. & Neville, A.M. (1977) A shorter immunoperoxidase technique for the demonstration of carcinoembryonic antigen and other cell products. Journal of Clinical Pathology, 30, 138-140.

188. Heyderman, E. (1981)
Journal of Clinical Pathology, 33,

189. Mason, D.Y. & Sammons, R.E. (1978) Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. Journal of Clinical Pathology, 31, 454-462.

190. Rathlev, T., Hocko, J.M., Franks, G.F., Suffin, S.C., O'Donnell, C.M. & Porter, D.D. (1981) Glucose

oxidase immunoenzyme methodology as a substitute for fluorescence microscopy in the clinical laboratory. Clinical Chemistry, 27, 1513-1515.

191. Streefkerk, J.G. (1972) Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol. Journal of Histochemistry and Cytochemistry, 20, 829-831.

192. Bulman, A.S. & Heyderman, E. (1981) Alkaline phosphatase for immunocytochemical labelling: problems with endogenous enzyme activity. Journal of Clinical Pathology, 34, 1349-1351.

193. Ponder, B.A. & Wilkinson, M.M. (1981) Inhibition of endogenous issue alkaline phosphatase with the use of alkaline phosphatase conjugates in immunohistochemistry. Journal of Histochemistry and Cytochemistry, 29, 981-984.

194. Avrameas, S., Indirect immunoenzyme techniques for the detection of antigens. Immunochemistry, 6, 825-831.

195. Sternberger, L.A. (1970) The unlabelled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase antihorseradish peroxidase) and its use in identification of spirochaetes. Journal of Histochemistry and Cytochemistry, 18, 315-333.

196. Guesdon, J-L., Terynck, D. & Avrameas, S. (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. Journal of Histochemistry and Cytochemistry, 27, 1131-1139.

197. Hsu, S.M., Raine, L. & Fanger, H. (1981) Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques. Journal of Histochemistry and Cytochemistry, 29, 581-584.

198. Yolken, R.H., Leister, F.J., Whitcomb, L.S & Santosham, M. (1983) Enzyme immunoassays for the detec-

tion of bacterial antigens using biotin labelled antibody and peroxidase biotin-avidin complex. Journal of Immunological Methods, 56, 319-327.

199. Hsu, S-M., Raine, L. & Fanger, H. (1981) A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. American Journal of Clinical Pathology, 75, 734-738.

200. Hsu, S-M, Raine, L. & Fanger, H. (1981) The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase techniques. American Journal of Clinical Pathology, 75, 816-821.

201. Chaiet, L. & Wolf, F.J. (1964) The properties of streptavidin, a biotin-binding protein produced by Streptomyces. Archives of Biochemistry and Biophysics, 106, 1-5.

202. Bonnard, C., Papermaster D.S. & Kraehenbuhl J.-P. (1984) The streptavidin-biotin bridge technique: Application in light and electron microscope immunocytochemistry. In: Immunolabelling for Electron Microscopy. Ed. Polak, J.M. & Varndell, I.M., Elsevier, Amsterdam.

203. Graham, R.C. & Karnovsky, M.J. (1966) Early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. Journal of Histochemistry and Cytochemistry, 14, 291-302.

204. Graham, R.C. (1965) Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole. Journal of Histochemistry and Cytochemistry, 13, 150-152.

205. Nakane, P.K. (1968) Simultaneous localisation of multiple tissue antigens using the the peroxidase lbelled antibody method: a study on the pituitary gland of the rat. Journal of Histochemistry and

Cytochemistry, 16, 557-560.

206. Hanker, J.S., Yates, P.E., Metz, C.B. & Rustioni, A. (1977) A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. Histochemical Journal, 9, 789-792.

207. Mason, D.Y. (1985) Immunocytochemical labelling of monoclonal antibodies by the APAAP immunoalkaline phosphatase technique. In: Techniques in Immunocytochemistry, Volume 3, p. 25-42. Ed. Bullock, G.R & Petrusz, P. Academic Press.

208. Huang, S.N., Minassian, H. & More, J.D. (1976) Application of immunofluorescent staining on paraffin sections improved by trypsin digestion. Laboratory Investigation, 35, 383-390.

209. Curran, R.C. & Gregory, J. (1977) The unmasking of antigens in paraffin sections of tissue by trypsin. Experientia, 33, 1400-1401.

210. Pinkus, G.S. (1985) Optimal immunoreactivity of keratin proteins in formalin fixed paraffin embedded tissue requires preliminary trypsinisation. Journal of Histochemistry and Cytochemistry, 33, 465-473.

211. Mepham, B.L., Frater, W. & Mitchell, B.S. (1979) The use of proteolytic enzymes to improve immunoglobulin staining by the PAP technique. Histochemical Journal, 11, 345-357.

212. Sigma Chemical Company, PO Box 14508, St Louis, MO 63178, USA.

213. Taylor, C.R. (1985) Monoclonal antibodies and 'routine' paraffin sections. Archives of Pathology and Laboratory Medicine, 109, 115-116.

214. Larsson, L-I. (1984) Labelled antigen detection methods. In: Immunolabelling for Electron Microscopy, p. 123-128. Ed. Polak, J.M. & Varndell, I.M. Elsevier, Amsterdam.

215. Sternberger, L.A. (1979) Immunocytochemistry, 2nd

edn. John Wiley & Sons, New York.

216. Kohler, G. & Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256, 495-497.

217. Pulvertaft, R.J.V. (1965) A study of malignant tumours in Nigeria by short term tissue culture. Journal of Clinical Pathology, 18, 261-273.

218. Nonoyama, M. & Pagano, J.S. (1971) Detection of Epstein-Barr viral genome in non-productive cells. Nature New Biology, 233, 103-106.

219. Klein, G., Lindahl, T., Jondal, M., Leibold, W., Menezes, J., Nilsson, K. & Sundstrom, C. (1974) Continuous lymphoid cell lines with characteristics of B-cells, lacking the Epstein-Barr virus genome and derived from three human lymphomas. Proceedings of the National Academy of Science of the USA, 71, 3283-3286.

220. McLean, I.W. & Nakane, P.K. (1974) Periodate-lysine-paraformaldehyde fixative. A new fixative for electron microscopy. Journal of Histochemistry and Cytochemistry, 22, 1077-1083.

221. Morris, R.J. & Barber, P.C. (1983) Fixation of Thy-1 in nervous tissue for immunohistochemistry. Journal of Histochemistry and Cytochemistry, 31, 263-274.

222. Bourne, J.A. (1983) Handbook of Immunoperoxidase Staining Methods. DAKO Corporation, Denmark.

223. Szanto, P.B. A modified method for the removal of the nasopharynx and accompanying organs of the throat. Archives of Pathology, 38, 313-320.

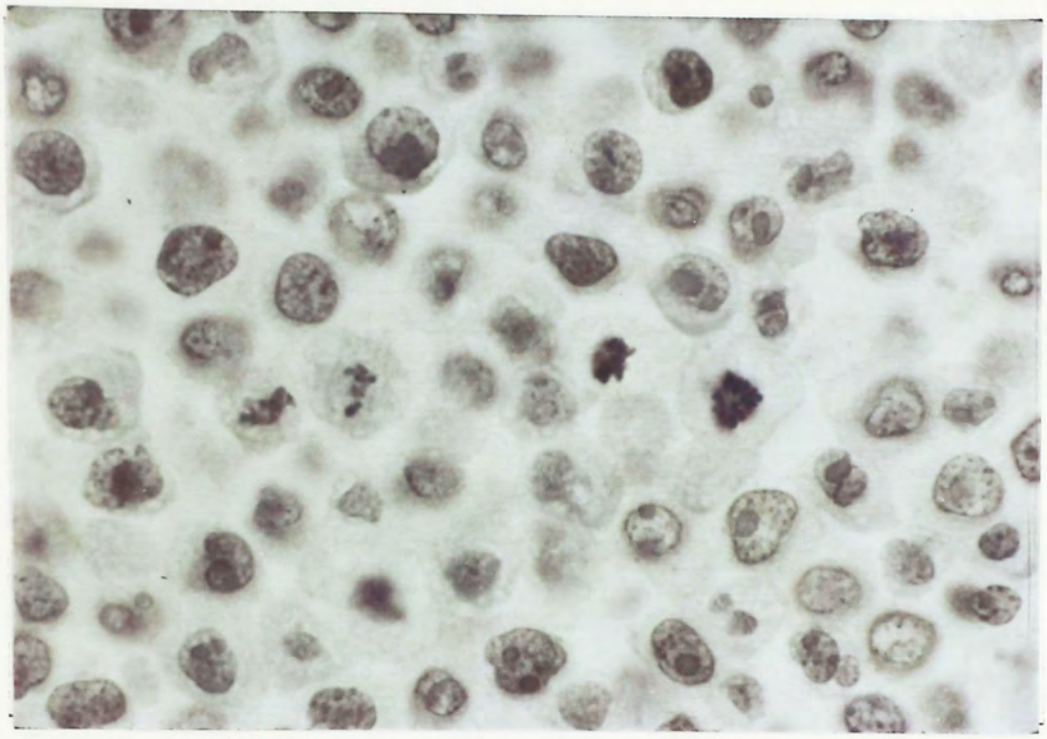
224. Henle, W., Guerra, A. & Henle, G. (1974) False negatives and prozone reactions in tests for antibodies to Epstein-Barr virus associated nuclear antigen. International Journal of Cancer, 13, 751-754.

225. Southgate, H.W. (1927) Note on preparing mucicarmine. Journal of Pathology and Bacteriology, 30, 729.

226. Cook, H.C. (1982) Carbohydrates. In: Theory and Practice of Histological Techniques, 2nd edn, p. 180-216. Ed. Bancroft, J.D. & Stevens, A. Churchill Livingstone, Edinburgh.
227. Bancroft, J.D. & Cook, H.C. (1984) Manual of histological techniques. Churchill Livingstone, Edinburgh.
228. Steedman, H.F. (1950) Alcian blue 8GS; a new stain for mucins. Quarterly Journal of Microscopic Science, 91, 477-479.
229. Mowry, R.W. (1958) Observations on the use of sulfuric acid ether for the sulfation of hydroxyl groups. Journal of Histochemistry and Cytochemistry, 6, 82-83.
230. Lendrum, A.C. (1947) The phloxin-tartrazine method as a general histological stain and for the demonstration of inclusion bodies. Journal of Pathology and Bacteriology, 59, 399-404.
231. Gordon, H. & Sweets, H.H. (1936) A simple method for the silver impregnation of reticulum. American Journal of Pathology, 12, 545-551.
232. Hybritech Inc., 11095, Torreyana Road, San Diego, California 92121, USA.
233. Amersham International PLC, Amersham Laboratories, White Lion Road, Amersham, Bucks HP7 9LL, England.
234. Dakopatts a/s, 42, Produktionsvej, Glostrup, DK 2600, Denmark.
235. Lane, E.B. (1982) Monoclonal antibodies provide specific intramolecular markers for the study of epithelial tonofilament organisation. Journal of Cell Biology, 92, 665-673.
236. Nagle, R.B., Moll, R., Weidauer, H., Nemetschek, H. & Franke, W.W. (1985) Different patterns of cytokeratin expression in the normal epithelia of the upper respiratory tract. Differentiation, 30, 130-140.

FIGURE 9. Raji cells, acetone-methanol fixed and paraffin processed. EBNA is demonstrated as brown granularity in the nuclei. Modified complement anti-complement test. X1000.

FIGURE 10. A86-76. Stratified squamous epithelium. The constantly dividing basal cells are cuboidal with dark staining nuclei. As they mature, the cells move towards the surface, become flattened and their nuclei become small and pyknotic. The epithelium is separated from the deeper lamina propria by a basal lamina. Haematoxylin-eosin. X400.



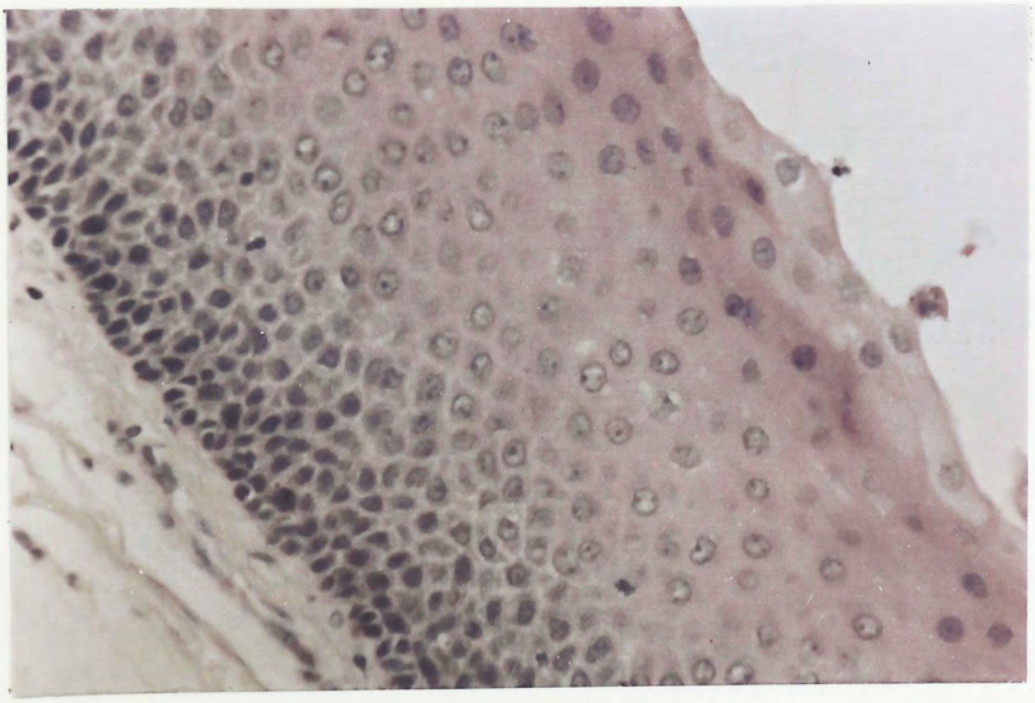
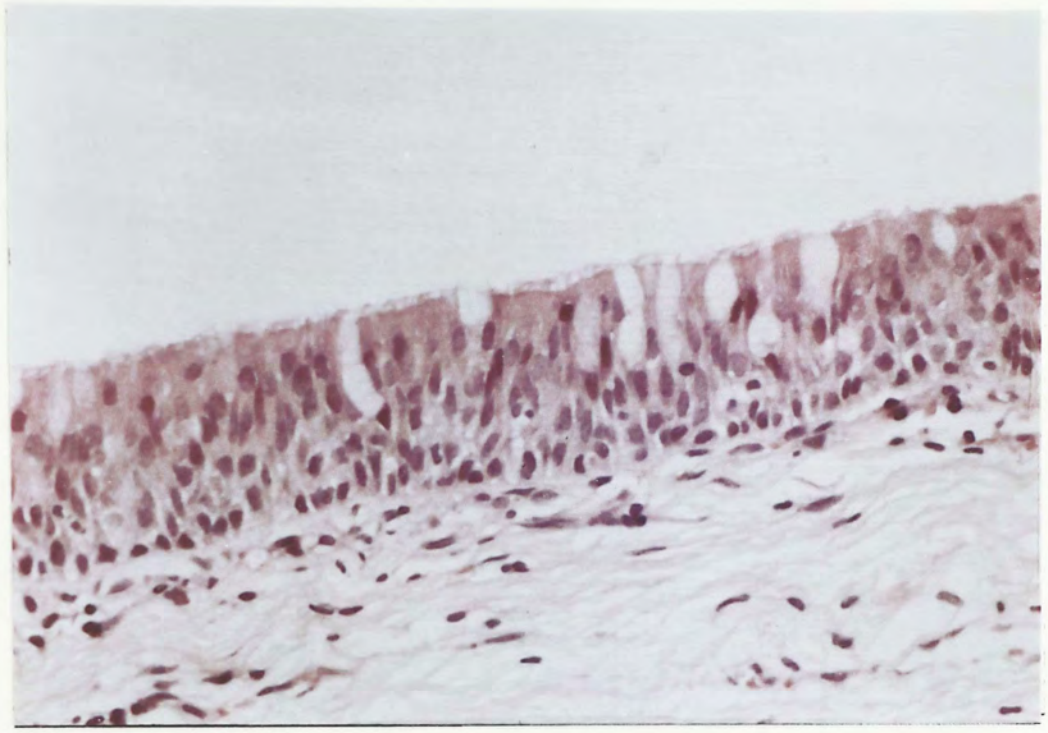


FIGURE 11. A87-371. Pseudostratified ciliated columnar epithelium. A single layer of elongated cells, with nuclei lying at different levels, giving the impression that there are several cell layers. There are many mucin producing goblet cells. The cell borders are ciliated. Haematoxylin-eosin. X400.

FIGURE 12. A87-371. Pseudostratified ciliated columnar epithelium. Acid mucins in the goblet cells are stained red. Southgate's mucicarmine. X400.



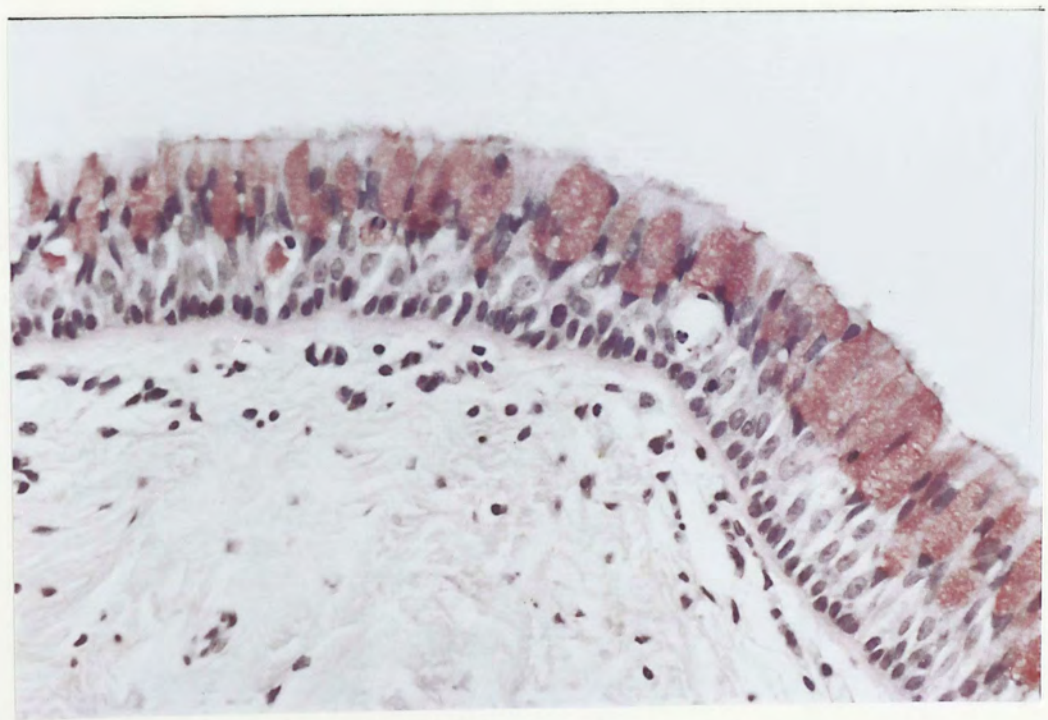
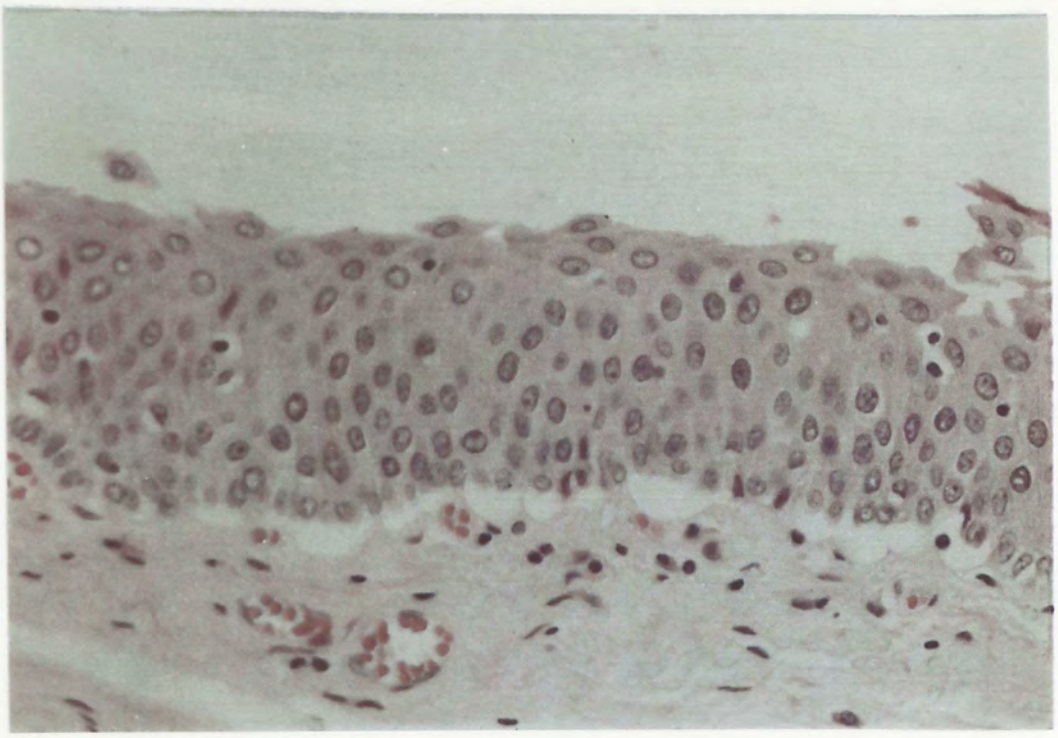


FIGURE 13. A87-371. Intermediate epithelium. A stratified epithelium of approximately 8 layers of cuboidal shaped cells. There is no flattening of the surface layers as is seen in squamous epithelium. Haematoxylin-eosin. X400.

FIGURE 14. A87-371. Intermediate epithelium. Higher magnification. Haematoxylin-eosin. X1000.



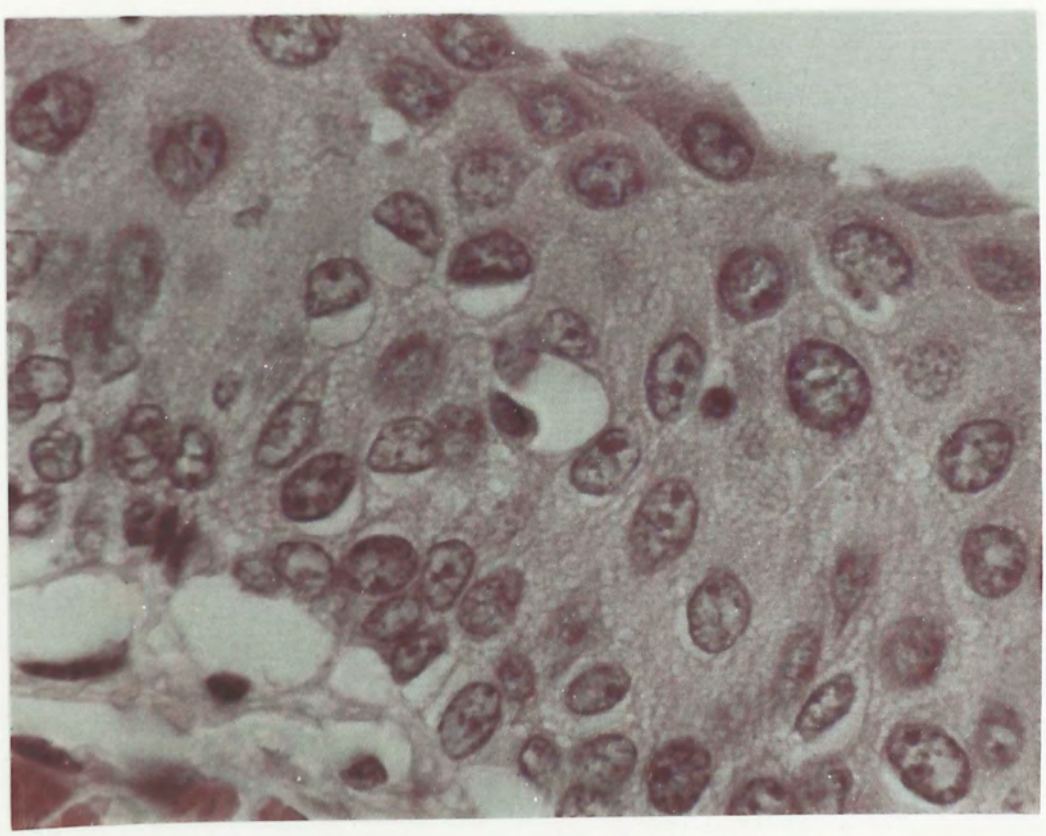
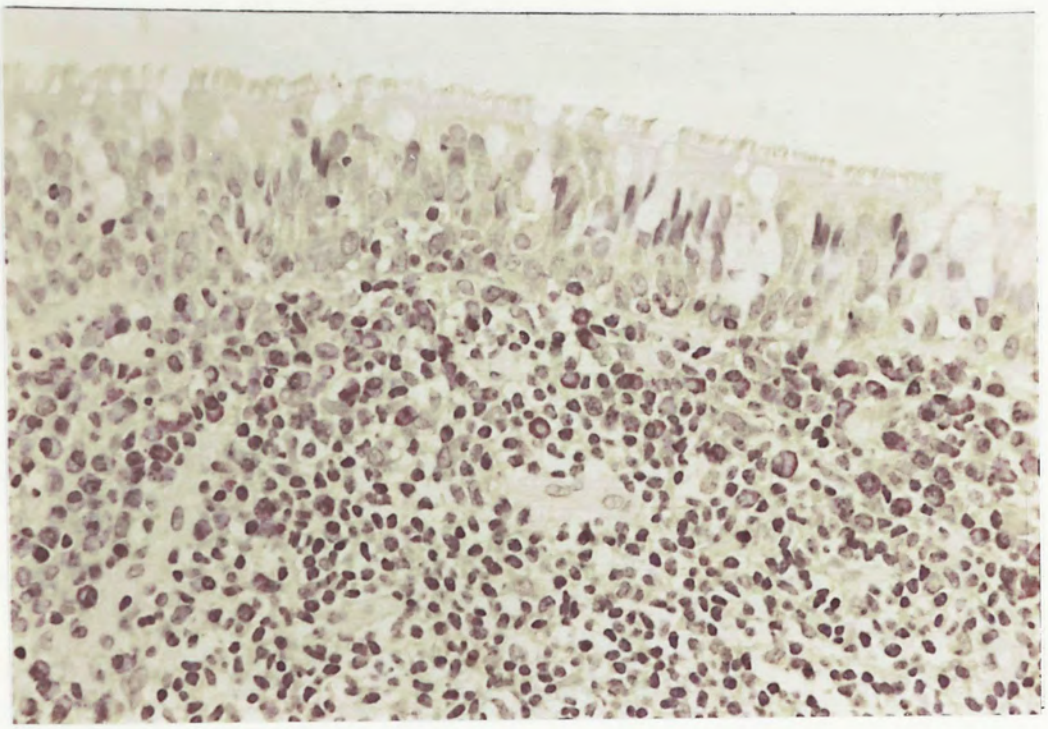


FIGURE 15. 86-4810 II. Pseudostratified columnar epithelium. Immunostained with MA6 antibody. Only lymphocytes in the lamina propria are heavily stained. X400.

FIGURE 16. 86-1162 I. Stratified squamous epithelium at the top, and intermediate epithelium at the bottom of the field. Immunostained with AE1/AE3. Only the deeper third of the squamous epithelium is stained, while the full thickness of the intermediate epithelium is stained. X 200.



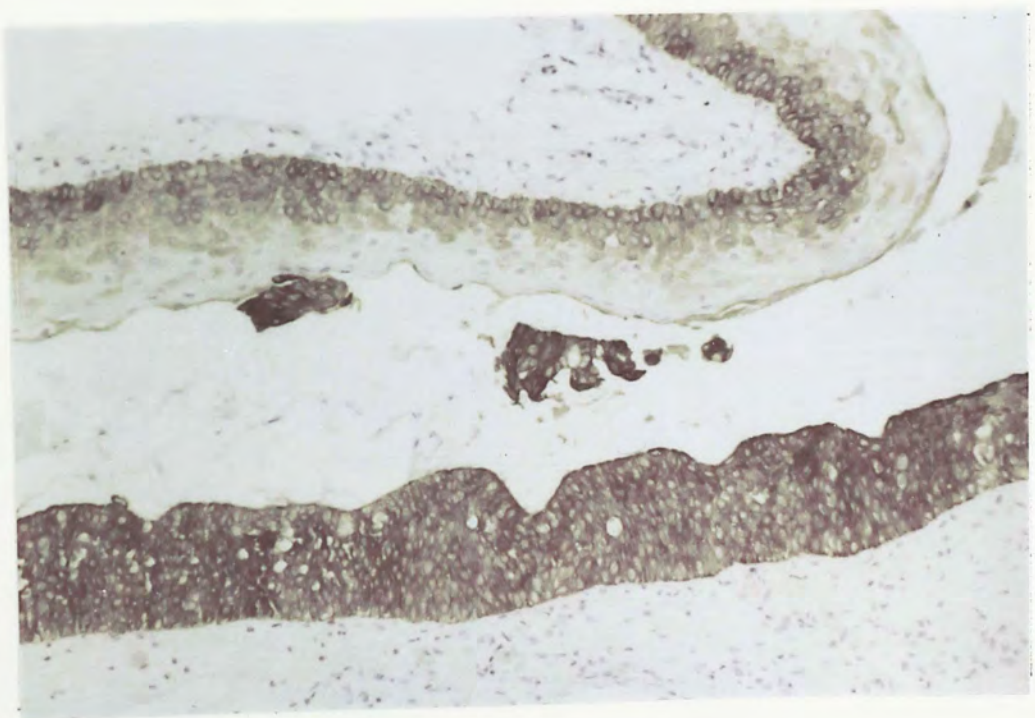


FIGURE 17. A87-368. High power view of stratified squamous epithelium. Approximately the lower third of cells are stained. AE1/AE3. X400.

FIGURE 18. 86-6445. Intermediate epithelium is negative when immunostained with anti cytokeratin 18. X400.

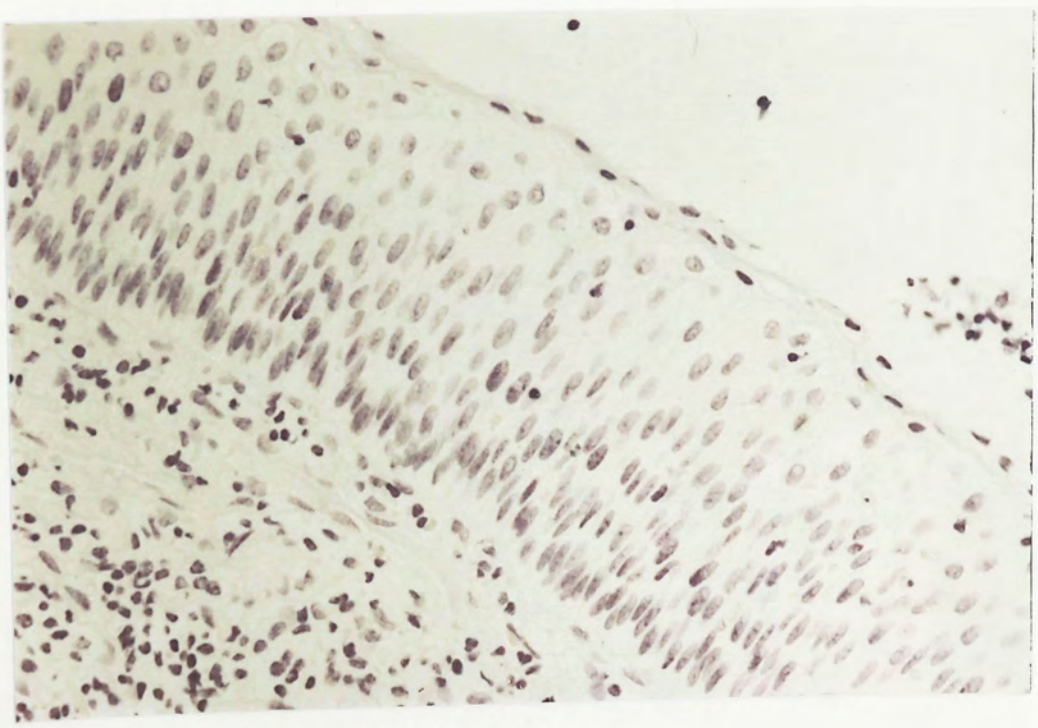
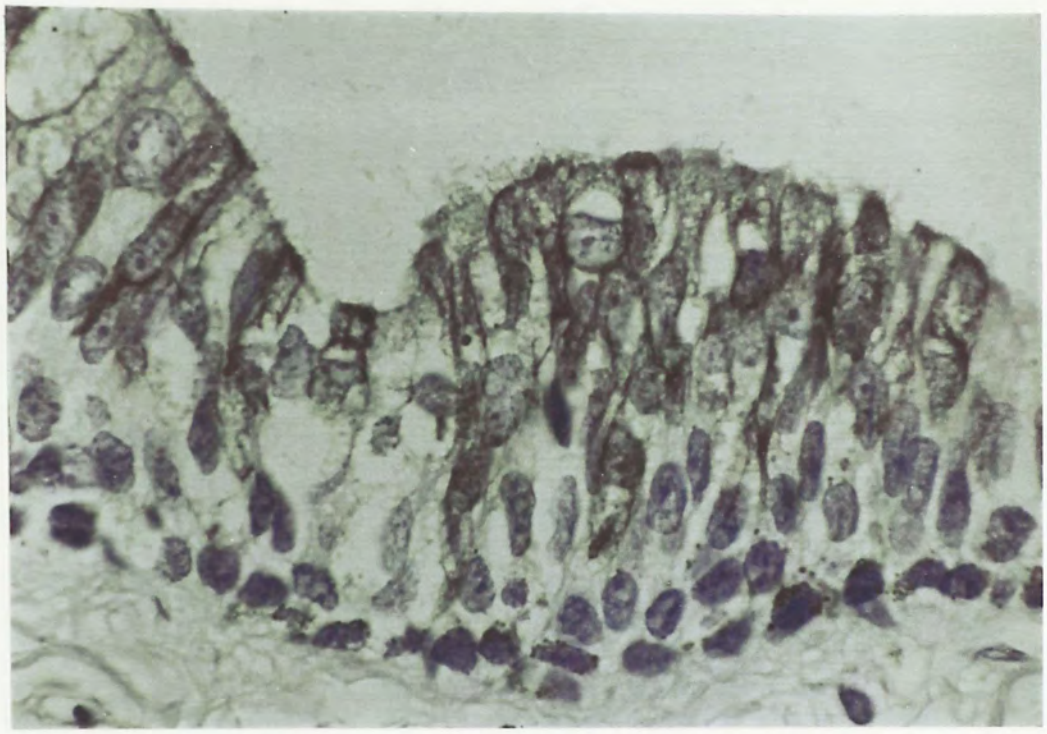


FIGURE 19. A87-368. Columnar epithelium immunostained with anti cytokeratin 18. The upper parts of the cells are heavily stained, while the deeper parts are unstained. X1000.

FIGURE 20. 86-1788 II. The pale staining area in the centre of the field shows squamous metaplasia, while on either side is darkly staining intermediate epithelium. AE1/AE3. X400.



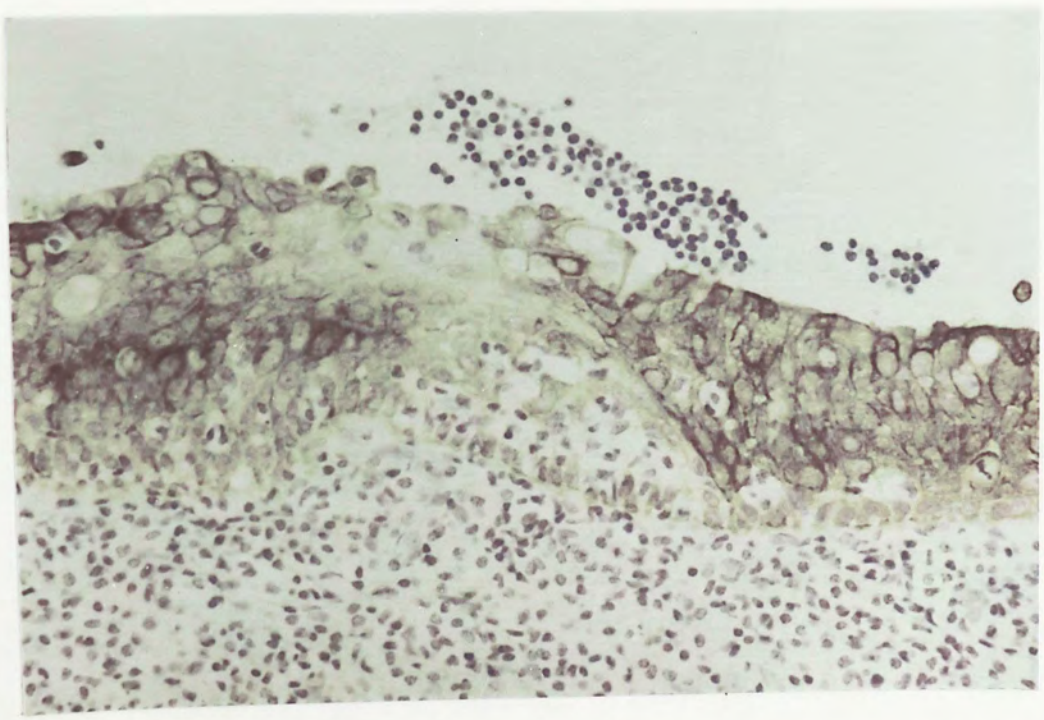
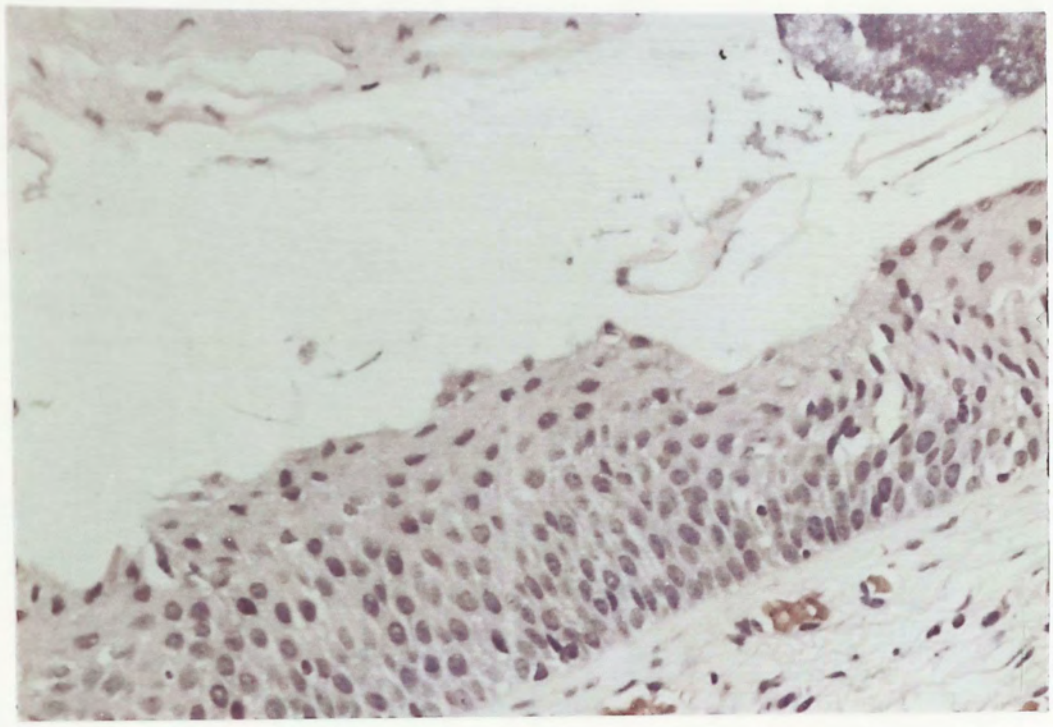


FIGURE 21. 86-1162 I. Intermediate epithelium with patches of squamous metaplasia which are difficult to identify in haematoxylin-eosin stained preparations. X400.

FIGURE 22. 86-1162 I. Same field as figure 22 immunostained with AE1/AE3. The pale areas show squamous metaplasia. X400.



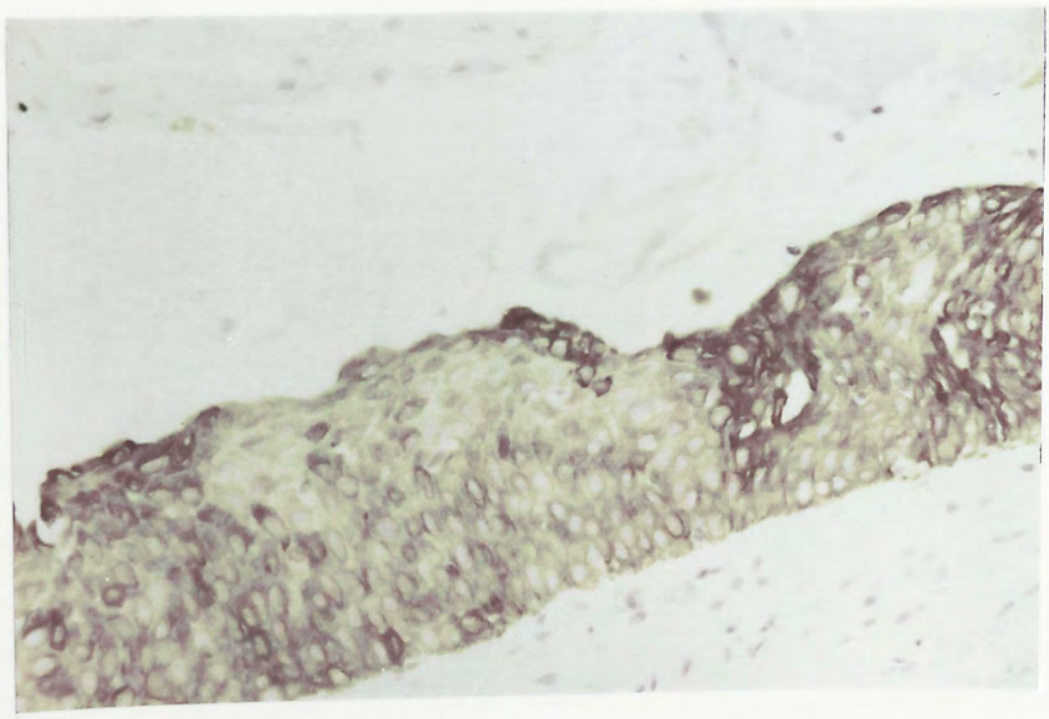
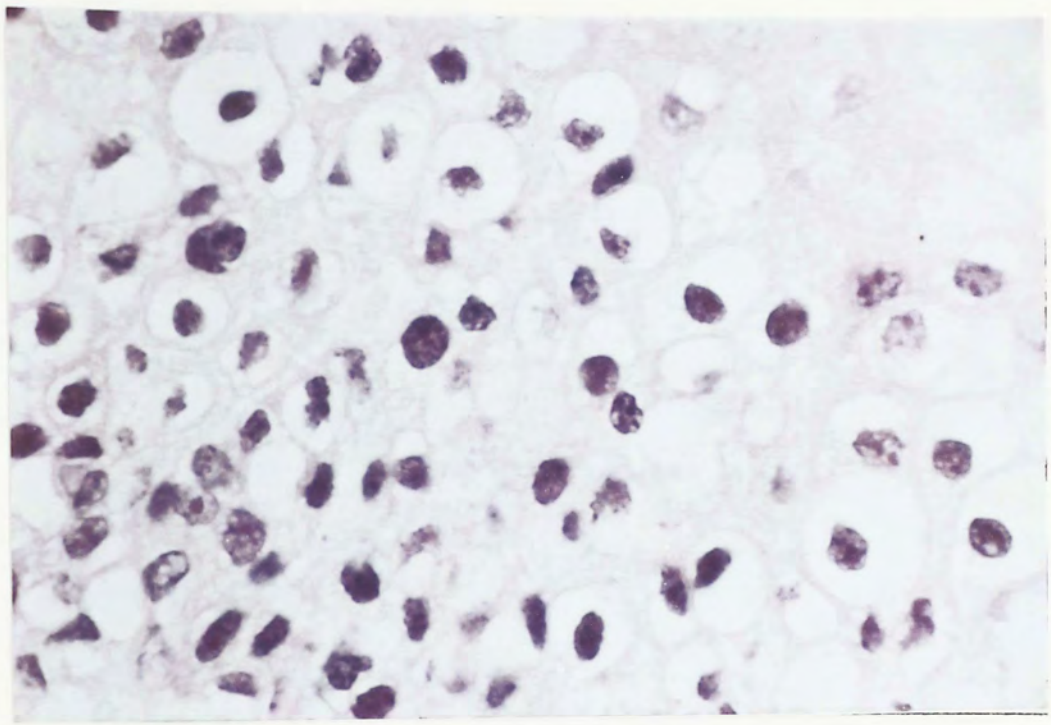


FIGURE 23. 85-4370 I. Squamous epithelium containing koilocytes which have a perinuclear halo and cytoplasmic vacuolation. Also see figure 14. X1000.

FIGURE 24. 85-3183. Squamous epithelium with koilocytes. Immunostained with AE1/AE3. The koilocytes are unstained. X1000.



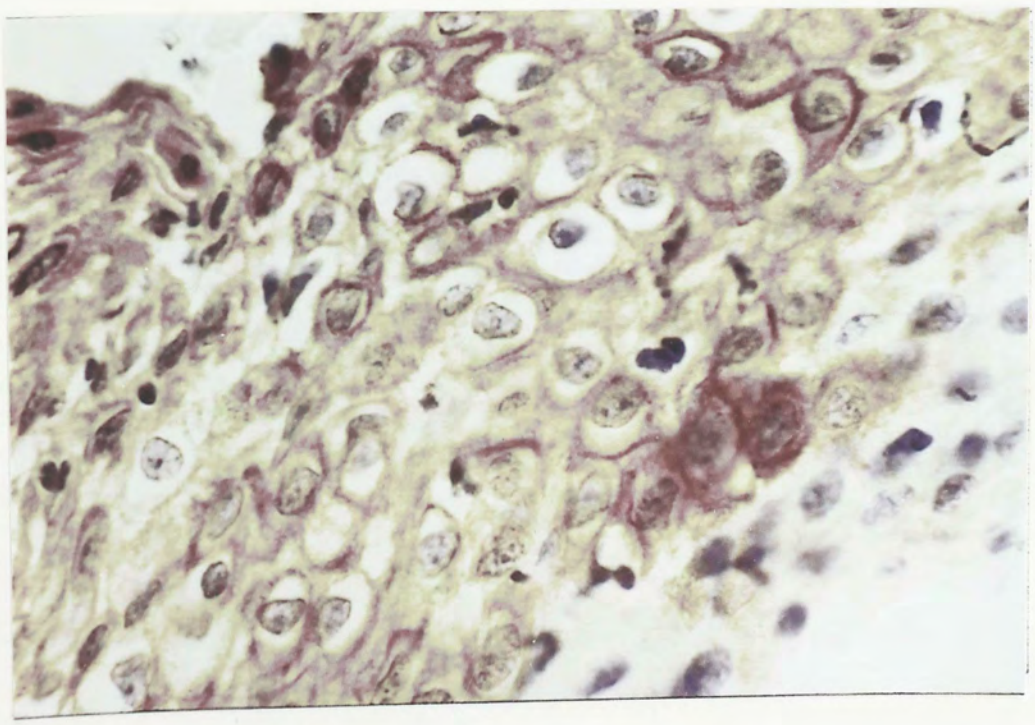
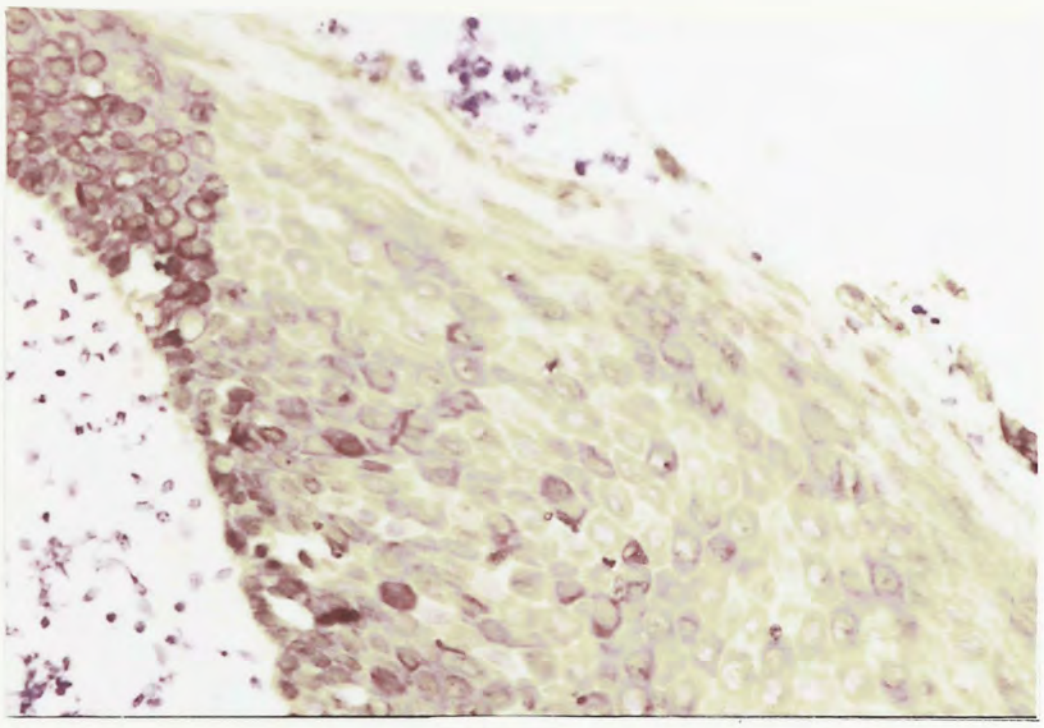


FIGURE 25. 85-2348 II. Squamous epithelium with a proliferation of disordered cells showing hyperplasia and intraepithelial neoplasia which is stained pale. Relatively normal dark staining epithelium in the top left hand corner. AE1/AE3. X400.

FIGURE 26. 86-5120 I. Undifferentiated nasopharyngeal carcinoma. Parts of three islets of tumour cells are clearly encapsulated by black reticulin fibres. Gordon and Sweets' silver impregnation. X100.



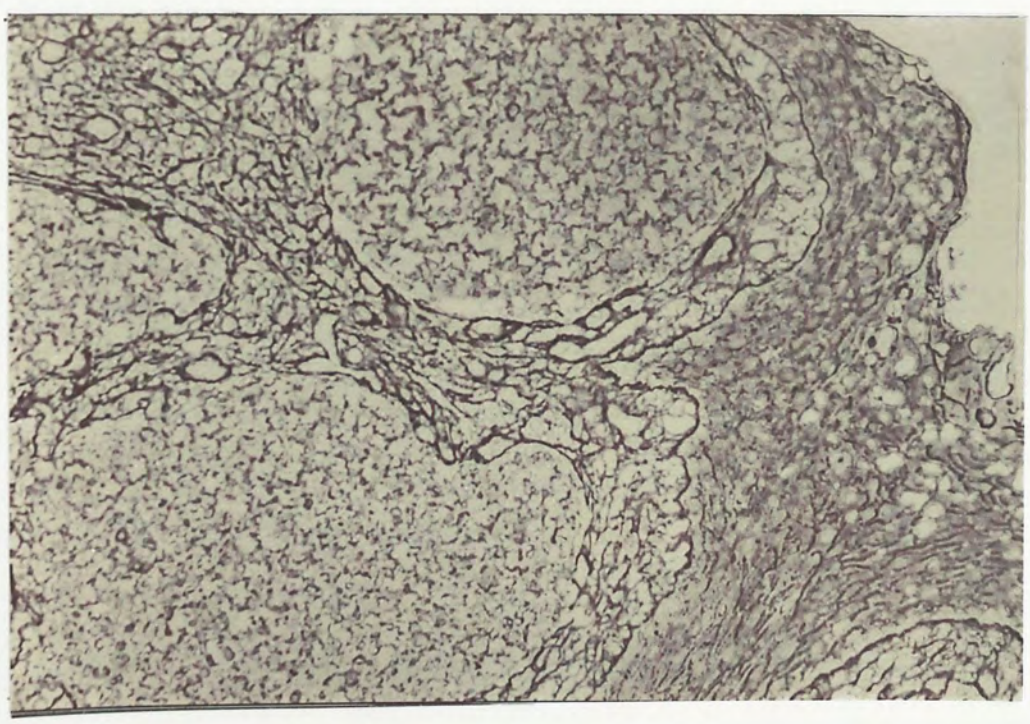
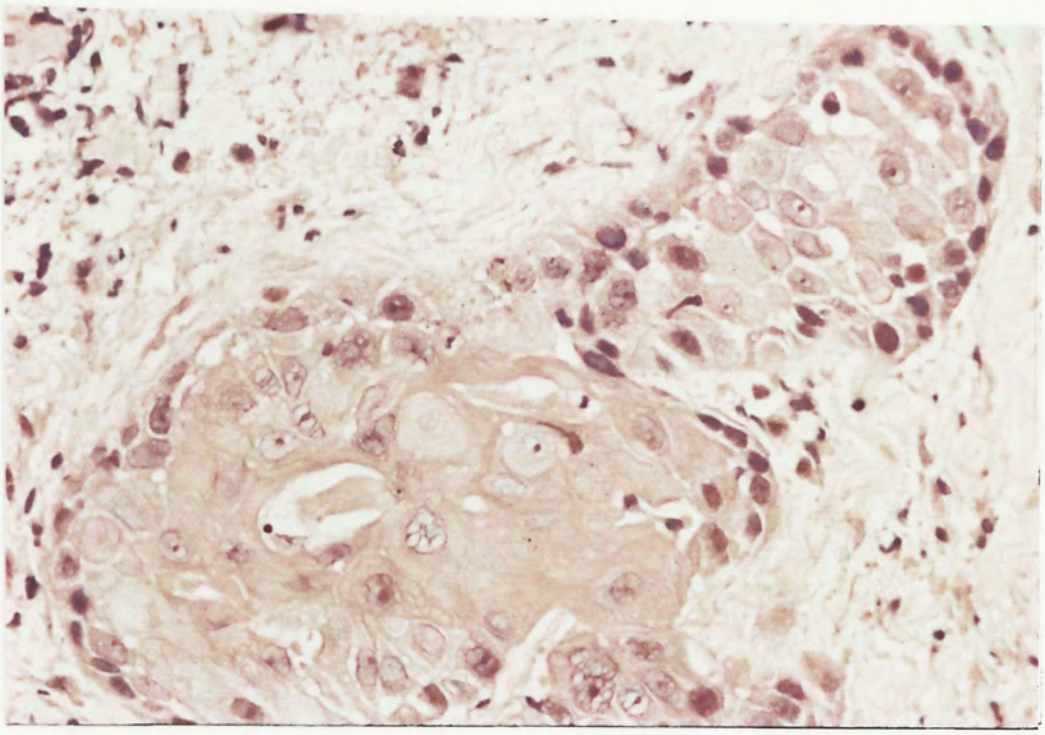


FIGURE 27. 87-443. Keratinising squamous cell carcinoma. A clump of tumour cells surrounded by a connective tissue stroma. The tumour cells are showing some pink-staining keratin. Haematoxylin-eosin. X400.

FIGURE 28. 87-443. Same case as figure 27. Keratin is stained red with phloxine tartrazine method. X400.



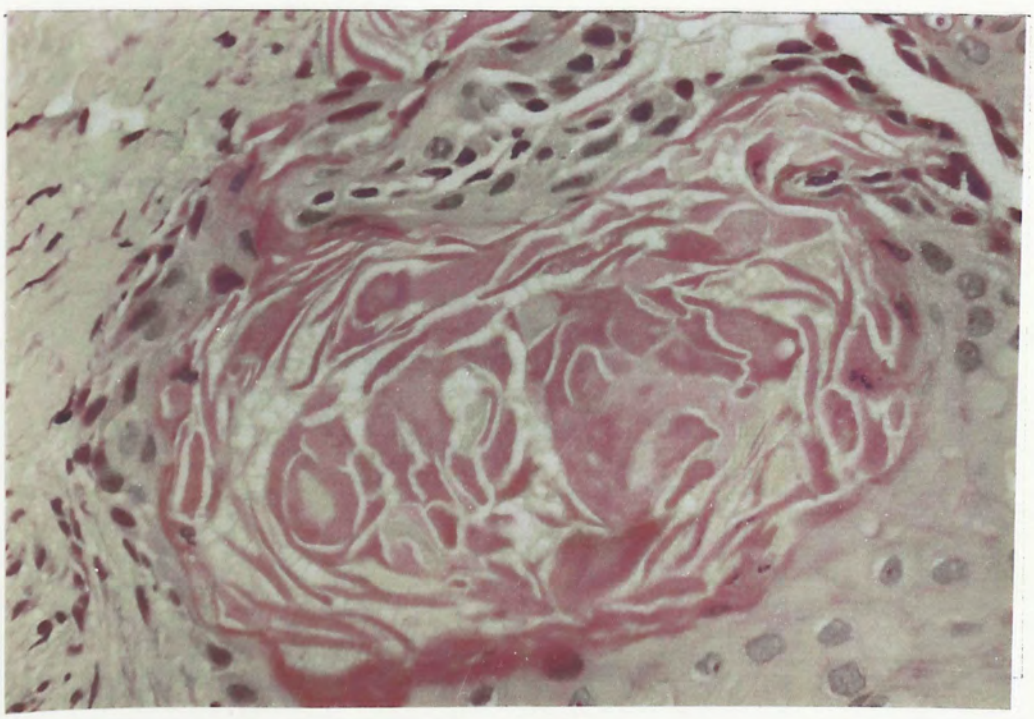
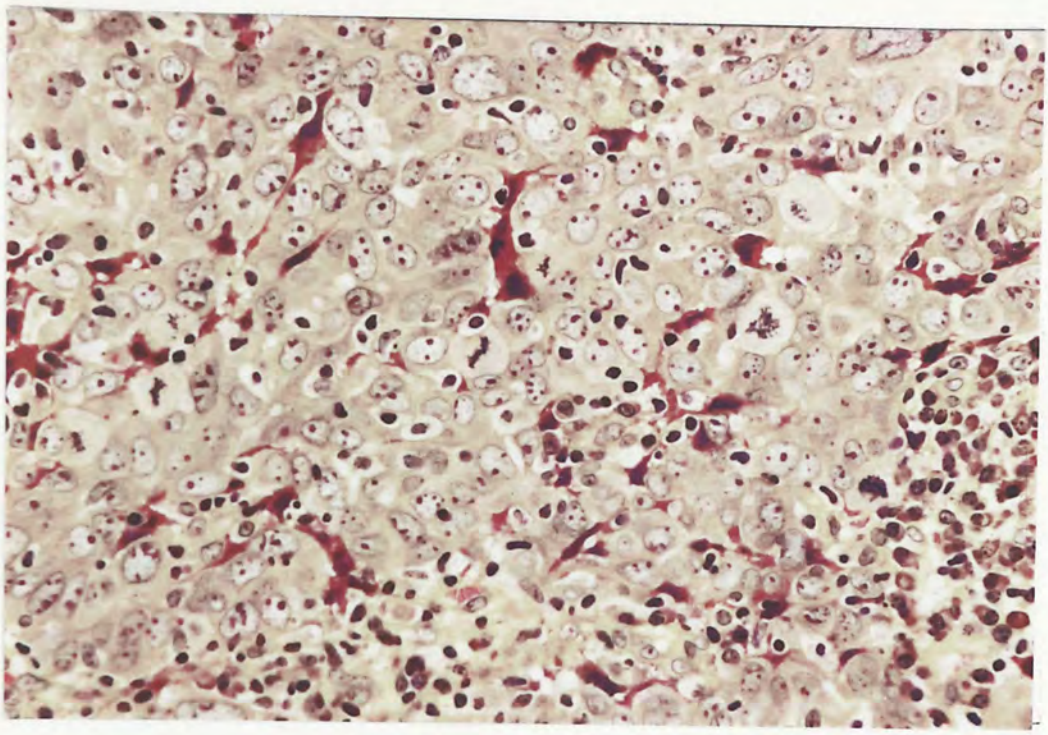


FIGURE 29. 86-5120 I. Undifferentiated nasopharyngeal carcinoma. Nucleoli are prominent. Several mitoses can be seen. Phloxine tartrazine. X400.

FIGURE 30. 85-831 I. Undifferentiated nasopharyngeal carcinoma. An islet of tumour cells can be seen in the centre of the field, amongst a dense lymphocytic infiltrate. Haematoxylin-eosin. X400.



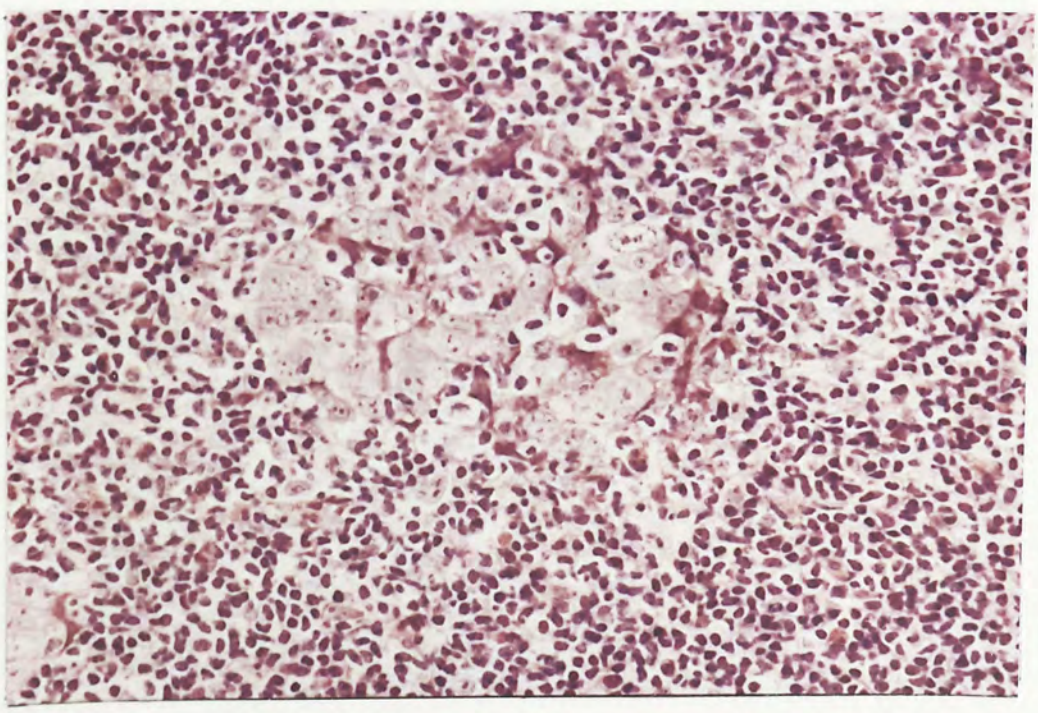
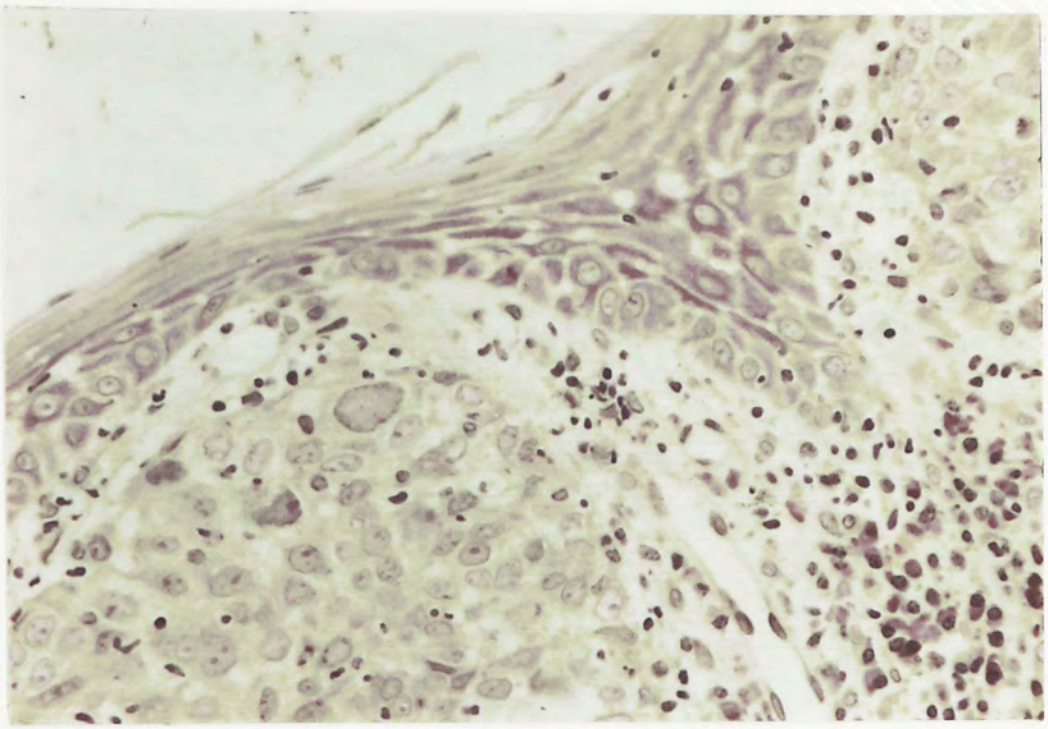


FIGURE 31. 86-5020 I. Undifferentiated nasopharyngeal carcinoma immunostained with MA6. Lymphocytes are heavily stained, squamous epithelium is moderately stained while staining of the tumour is pale. X400.

FIGURE 32. 87-443. Same case as figures 27 and 28. Tumour cells stain clearly with AE1/AE3. X400.



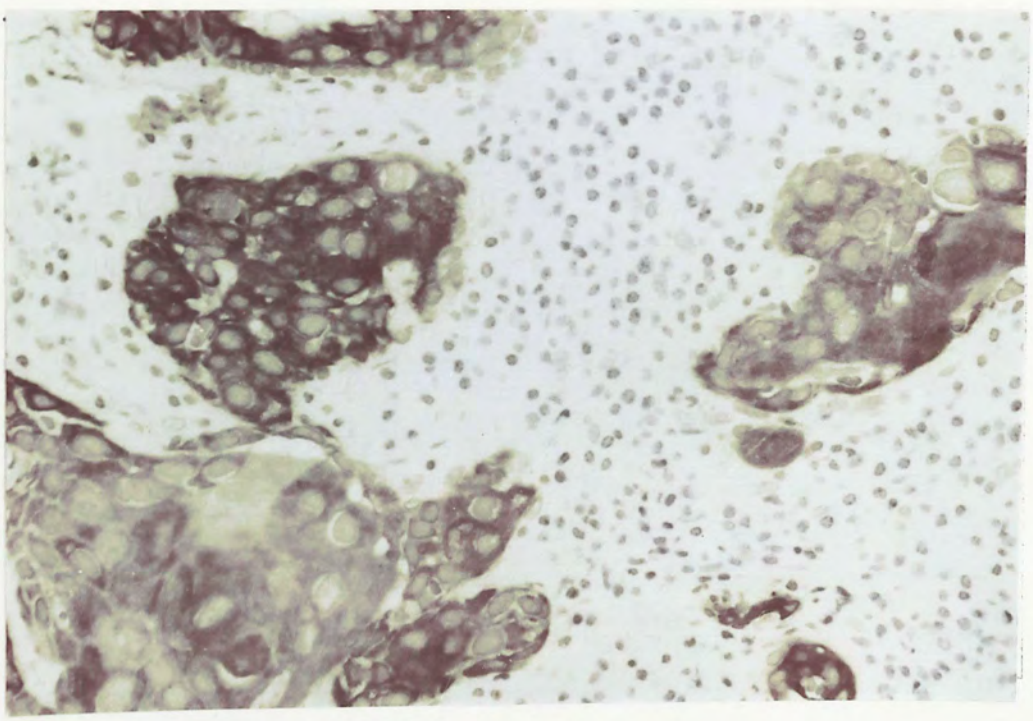


FIGURE 33. 86-4074 I. Undifferentiated nasopharyngeal carcinoma. Tumour cells are clearly stained against the connective tissue stroma and lymphocyte infiltrate. AE1/AE3. X400.

FIGURE 34. 86-8695 I. Undifferentiated nasopharyngeal carcinoma again is clearly demonstrated. AE1/AE3. X400.